UNIVERSITY of TASMANIA

The effect of L-tryptophan on aggressive interactions in barramundi (*Lates calcarifer)*, and food intake of Atlantic salmon (*Salmo salar)* during seawater transfer

Digory Hulse

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

University of Tasmania

February 2017

Declaration

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis. To the best of my knowledge and belief this thesis contains no material previously published or written by another person except where due acknowledgement is made in the text.

Digory Hulse

Authority of Access

The thesis may be made available for loan and limited copying in accordance with the *Copyright Act 1968.*

Digory Hulse

Abstract

Aggressive behaviour between fish is common in both freshwater and seawater environments, at all stages of development and both between species and within species groups. In monoculture fish farms intracohort aggressive behaviours result in the establishment of dominance and feeding hierarchies, growth depensation (the increase in size variability within a population over time due to differences in growth rates), stress, injury, increased pathogen susceptibility and death. Given the material impact of aggressive behaviours on stock value, Australian fish farmers of both Atlantic salmon and barramundi, carry out specific husbandry practices and provide culture environments that aim to reduce these interactions.

Tryptophan (TRP), an essential amino acid, is the precursor of serotonin (5-HT), a monoamine neurotransmitter implicated in mood modulation and behavioural change. This study examined whether supplementary dietary tryptophan (TRP) reduces the rate of aggressive interactions between barramundi. It also examined whether supplementary dietary tryptophan causes a quicker resumption of feeding in Atlantic salmon smolt post-seawater transfer (SWT).. These research questions were considered of sufficient commercial interest to warrant sponsorship of this study by an Australian aqua feed manufacturer.

This study involved 5 experiments: (1) the effect of supplementary dietary TRP on the behavioural response of a resident barramundi to a smaller intruder; (2) the behavioural response of isolated barramundi to their reflection, a foreign object , and a smaller intruder, with measurement of physiological stress responses and brain serotonergic activity; (3) the effect of supplementary dietary TRP on growth performance, cannibalism, physiological stress response and brain serotonergic activity in groups of barramundi fed to satiation; (4) the effect of supplementary dietary TRP on growth performance, cannibalism, physiological stress response and brain serotonergic activity in groups of barramundi fed a restricted ration; (5) the effect of supplementary dietary TRP on resumption of feeding post-SWT, growth performance, physiological stress response and brain serotonergic activity in groups of Atlantic salmon smolt fed to satiation.

In experiment 1, aggressive behaviour of a resident barramundi, fed either a reference feed or one supplemented with TRP at 19.4 $mg.g^{-1}$ total inclusion, toward a 50% smaller (length) conspecific intruder was examined. Intruder fish were confined with residents for 24 hours after the residents had been fed the experimental feeds for 1, 2, 3, 4, 5, 6, 7, 8 or 14 days. Video data were analysed for 5 minutes of each hour over the 24 hour period and behaviours were presented for the first 5 minutes of confinement, the first 6 hours of confinement, and the full 24 hours of confinement. Data for the full 24 hours of confinement are presented for only days 1 and 14. No differences in behavior were found between these treatments during the first 5 minutes of confinement for any day.

In relation to the 6 hours of confinement no differences in behavior were observed on all but 2 days tested. On day 14 more attacks were perpetrated on the intruder by fish fed the reference feed. On day 6 a greater latency to chasing by resident fish was observed in the treatment fed the reference diet compared to those fed the TRP supplemented feed and a greater latency to eyeballing by resident fish was observed for fish fed TRP supplemented feed compared to those fed the reference feed.

Across 24 hours of confinement on day 14 more attacks were perpetrated on the intruder by fish fed the reference feed. A greater latency to eyeballing by resident fish was observed on day 1 over 24 hours of confinement for fish fed TRP supplemented feed compared to those fed the reference feed.

No differences in survival of intruders were observed at either initial confinement, or after 6 or 24 hours of confinement between feed treatments.

Experiment 2 aimed to identify behavioural types in barramundi by comparing individual responses to 3 different stimuli with serotonergic and physiological stress responses. Stepwise regression was used to identify predictor variables of behavioural response. Solitary resident barramundi were acclimated to test chambers and exposed to each of the 3 stimuli (reflection, foreign object and intruder) for 1 hour, with a 72 hour gap between tests to reduce the possible behavioural effect of one test upon the next. After the final test (the intruder test) resident fish were sampled for blood and brain tissue.

Eyeballing by residents was found to be the strongest predictor of attack by residents on intruders. Eyeballing the foreign object was the strongest predictor for chasing the intruder. Blood glucose was elevated in resident fish chased by intruders, however was not significantly elevated in residents that chased the intruder. Due to a processing error data for serotonergic activity were not available.

In experiments 3 and 4 growth performance, rates of cannibalism, stress physiology and serotonergic activity were examined. Across the two experiments fish were offered feed supplemented with TRP at 4.6, 19, 21.4, 23.8, 28.0, 31.0, 33.6, 40.9 $mg.g^{-1}$ (Exp 3); and 4.6, 14.8, 15.9 and 19.0 $mg.g^{-1}$ (Exp 4). Fish were fed to satiation in Exp 3, and to either satiation or 50% of satiation in Exp 4.

Supplementary dietary TRP was found to inhibit food intake in barramundi in a dose dependent manner, and specific growth rate was negatively correlated with increased dose. When barramundi were offered a range of TRP supplemented feeds to satiation, fish fed the reference feed, and the feed with the least supplementary TRP had more efficient feed conversion ratios. No differences in feed conversion ratio were observed between barramundi offered either a satiation or 50% of satiation ration at any of the TRP inclusions, despite specific growth rate being strongly affected by ration. Neither ration size nor TRP content affected survival or physiological stress response measured as whole body cortisol, and whole blood glucose and lactate.

Supplementary dietary TRP (4.9, 10.9, 21.8, and 46.3 $mg.g^{-1}$ of feed) was found to inhibit food intake in Atlantic salmon smolt in a dose dependent manner prior to seawater transfer in Exp 5. Seawater transfer of Atlantic salmon smolt reduced food intake for both high (period of low water level followed by immersion in seawater) and low (slow flooding of tanks with seawater) stress transfer types and for all feed types (4.9, 10.9, 21.8, and 46.3 $mg.g^{-1}$ of feed). Supplementation with TRP did not increase the level of food intake or reduce the period of reduced food intake post-seawater transfer. Neither supplementation of feed with TRP, nor seawater transfer type affected growth or the feed conversion ratio. Serum cortisol was increased by seawater transfer however no differences in serum cortisol response were

observed between seawater transfer type. Serum osmolality values were higher for Atlantic salmon subjected to high stress seawater transfer compared to low stress seawater transfer. Brain TRP concentration was higher post-seawater transfer in fish fed the reference feed and subjected to high stress transfer compared to those subjected to low stress transfer.

Experiments show that supplementary dietary TRP, at the inclusion levels used in the current study, in general do not significantly inhibit behavioural interactions (measured either directly by video or by the proxy of relative growth performance) between barramundi or Atlantic salmon. The non-feeding period observed in Atlantic salmon smolt post-seawater transfer was not alleviated by supplementing food with TRP. Furthermore the strong hypophagic effect of TRP further reduces its potential as a functional ingredient for practical application in fin fish food.

This thesis discusses possible pathways for the observed hypophagic action of dietary TRP, as well as the apparent lack of behavioural modulation. The attribution of TRP, serotonin (5-HT), 5-hydroxyindoleacetic acid (5-OHIAA) or the ratio 5-HT : 5-OHIAA, to food intake and behavioural observations are discussed in context with sometimes contrary findings of other studies. Associations between serotonergic activity and HPI axis activation are examined.

All aspects of the study conformed to the requirements of the NHMRC *Australian code of practice for the care and use of animals for scientific purposes 8th edition 2013*, and more specifically to the UTAS Animal Ethics Committee approved project AOO14598.

Acknowledgements

Many thanks are due to my supervisory team, Associate Professor John Purser, Dr Mark Adams, and Dr Ryan Wilkinson, for their guidance throughout the course of this study. Thank you for steering me patiently and wisely toward the culmination of this work.

Without the industry support provided by Ridley Aqua Feeds this project would not have been possible. Therefore I would like to thank Dr Mark Porter for being enthusiastic about the initial collaboration, and Dr Richard Smullen for delivering continued support to the project. Many thanks to Arthur Poligeros of West Beach Aquaculture for the supply, and airfreighting of tightly size graded barramundi for the experiments of Chapter 2 and 3. Similarly thanks are due to Tassal for the supply of Atlantic salmon smolt for the experiment in Chapter 4.

There are lots of people from IMAS who helped with sampling, most of them numerous times, and without whom the process would have been impossible and not nearly so enjoyable. They include Ryan Wilkinson, Daniel Pountney, Kaeden Leonard, Mark Blumhardt, Mark Adams, Deborah Leonard, Victoria Valdenegro, Gianluca Amoroso, Martin Grunenwald, Bikram Gosht, Dinh Tran, Laura Gonzalez Poblete, Chris Mabin and Duc Nguyen. Discussions about statistics were usually not so enjoyable, however many thanks are still due to Leon Barmuta, Natalie Moltschaniwskyj, Elkana Ngwenya, Masayuki Tatsumi, Rebecca Mueller and Chris Mabin. Many thanks are given to David Nichols for his solid assistance with HPLC quantitation of brain analytes. Many, many thanks are given to Chris Mabin for sacrificing his precious time to play often hilarious games of table tennis over the last year. They pulled me through some dark days.

And finally, an enormous thank you to my four beautiful girls; my wife Sally and my daughters Aponi, Charlie and Chilli. Sally, thank you for putting up with this extended foray in to the study of aquaculture. It started in 2004, and now almost exactly 13 years to the day, a significant milestone has been reached. Thankyou for your unwavering support throughout. Thankyou to my wonderful children for putting up with me spending evenings and weekends in the office, and for occasionally being Mr Crankypants, when I should have been spending time with you.

Table of Contents

List of tables

List of figures

1 Chapter 1 - General Introduction

This research examines the effects supplementation of commercial type fish food with synthetic L-tryptophan (TRP) has on the Australian commercially important species Atlantic salmon (*Salmo salar*) and barramundi (*Lates calcarifer*). Since 2003 – 04 the largest increases in production value in the Australian aquaculture sector have been an AUD$358 million (194 %), and AUD$16 million (91 %) rise for salmonid and barramundi respectively. Both species display conspecific aggressive behaviours which can have negative impacts on production volume and value. Furthermore the natural process of transfer from fresh water to a marine environment for salmon triggers what is thought to be a stress induced period of greatly reduced food intake which in some instances is permanent. Therefore strategies to reduce aggressive encounters provide some mitigation to associated stock loss and consequent value depreciation, as well as being considered appropriate welfare measures. The use of dietary manipulation to achieve these ends is attractive as it involves minimal disturbance for the fish. The essential amino acid TRP present in the diet of all fish, enters the bloodstream and crosses the blood brain barrier, where it is the precursor to serotonin (5-HT). Numerous studies across a range of animal species have shown a behavioural impact of elevated 5-HT turnover in the brain, including a reduction in the rate of aggressive interactions and a mediation of the physiological stress response.

1.1 Aquaculture production

Aquaculture represents the fastest growing food production sector globally, expanding by 85% and 110% for tonnage and value respectively over the period 2000 to 2010 (FAO 2012). This expansion is driven by a burgeoning middle class in India and China with annual per capita fish consumption in developing regions increasing from 5.2 Kg in 1961 to 17.8 Kg in 2010 (FAO 2014). An additional driver may be the superior conversion of feed into muscle tissue of fish over warm blooded animals, with only 2% of consumed energy converted to biomass in homeotherms compared to 17% in poikilotherms (Smith and Smith 2009). World Global

aquaculture value reached US$ 144 billion in 2012 (FAO 2014), while aquaculture production, excluding aquatic plants, reached 66.6 million tonnes (T) in 2012, an increase of 6.9% from 2011. Oceania production, as a percentage of global production, has been consistently reported at approximately 0.3% since 1970. Aquaculture production (T) in the Oceania region is dominated by New Zealand (60%) and Australia (38%).

Australian aquaculture production, valued at AUD $1 billion accounted for 40% of the value of total domestic fisheries production, and 33% of the tonnage of total fisheries production in the year 2013 – 2014 (Savage and Hobsbawn 2015). Farmed salmonids accounted for 55% of total Australian aquaculture value at AUD $543 million. Tasmania produced 56% of the total value of Australian aquaculture production at AUD $559 million, of which salmonid production accounted for 91% (Savage and Hobsbawn 2015). Australian 2010 production per employee, a reflection of the degree of industrialisation of the industry, is approximately 30 T which is similar to Chile (35 T), greater than China (7 T) and India (4 T), however considerably less than North America (180 T) or Norway (187 T) (FAO 2012). The Australian industry is well positioned, given ample access to coastal water supplies, a stable governance structure and economy, combined with cautious development requirements and biosecurity practices, to significantly expand production and improve productivity over the forthcoming decade.

1.2 Aquafeeds

The most common feed type in commercial aquaculture is the extruded pellet. Extrusion involves ingredients being exposed to high temperature for a short period, which minimizes nutrient degradation while improving protein digestibility. The use of fishmeal and fish oil from marine products (wild harvested fish and shellfish) in animal feeds is a controversial topic. Historically a considerable quantity of marine product has been required for rendering to fishmeal and fish oil to satisfy the nutrition demands of farmed fish, and ensuring an end product high in omega 3. As the availability of cost effective sources of fishmeal have declined, financial and ethical pressures have resulted in a much more efficient and sustainable use of marine product. Global reduction fisheries (marine fishery products including by-catch

converted to fishmeal and fish oil) totaled 30.2 million tonnes in 1994; however that figure steadily decreased to 17.9 million tonnes in 2009 (FAO 2012). The corresponding production of fishmeal and fish oil has similarly decreased over the same time period at an annual average rate of 1.7 and 2.6% respectively (FAO 2012). As a percentage of the total capture the use of fish and shellfish for non-human food use, such as agricultural feed products, has declined from 37% in 1994 to 20% in 2009 (FAO 2012). The reduction can be partially attributed to the use of rejects from food fish, estimated at 6 million tonnes annually, which provides approximately 25% of all fishmeal production (FAO 2012). More importantly knowledge surrounding nutritional requirements and digestion of many species has increased with a concurrent increase in the inclusion of dietary fishmeal substitutes (FAO 2014). Furthermore, a reduction in waste is observed via reduced (improved) feed conversion ratios over the same period with predictions for further declines through to 2020 (FAO 2012).

There is a global impetus to reduce the quantity of fish meal and fish oil used in the manufacture of commercial fish feeds, whilst maintaining the appropriate dietary composition, health benefits and production efficiencies that these products afford. There are three core strategies that can deliver reductions in the fishmeal and fish oil requirements of compound aquafeeds; (1) focusing effort on herbivorous species, (2) improving the capacity for feed to be converted to tissue, and (3) replacing fishmeal protein and oils with plant based alternatives.

In line with the first strategy, Naylor *et al.* (2000) present data from 1997 which showed total aquaculture production to be 29 million T, of which 19 million T was the net gain to overall fishery production after fisheries capture for feeds is removed. The authors attribute this net gain to carp, milkfish and catfish fed primarily on herbivorous diets. Increases in the proportion of carnivorous species under culture, or a shift to the more intensive culture of omnivorous species, as a result of increasing production costs and land availability pressures, may have an impact on the net gain data. More recent data shows that the ratio of wild fish inputs to total farmed fish output reduced from 1.04:1 in 1995 to 0.6:1 in 2007 (Naylor *et al.* 2009).

The second strategy is made up of many strands. Significant improvements in feed conversion ratios have been achieved over the past two decades via research on the nutritional

requirements and digestive processes of many species, as well as how to best process raw ingredients to make them more suitable within feeds (Glencross *et al.* 2007). The selective breeding of plants commenced at the turn of the 20th century following the work of Mendel. The same processes were applied to terrestrial livestock shortly after, however organised selective breeding programs for fish did not occur until much later. Many aquaculture breeding programs have delivered high genetic gains as a result of high fecundity, short generation intervals and the high heritability of selected traits (Gjedrem *et al.* 2012). Over 5 generations of selection Atlantic salmon have shown genetic gains of 113% for growth rate and a 20% reduction in feed conversion ratio's (FCR) (Thodesen *et al.* 1999). Over 100 commercial breeding programs are reported to cover 25 species with an average number of 2.8 important traits selected (Gjedrem *et al.* 2012). Traits selected for are invariably fast growth rate, improved disease resistance and reduced FCR.

Thirdly, despite a decrease in reduction-fisheries production, and reduced fishmeal and fish oil production, aquafeeds for many species have traditionally included fishmeal at over 50% (Glencross *et al.* 2007). A heavy reliance on a single ingredient presents risks associated with quality, availability and cost. Furthermore the perception of aquaculture of carnivorous species is that it is a net consumer rather than producer of fish (Naylor *et al.* 2000). Over the past two decades considerable research has been conducted into alternative sources of protein and lipid for aquafeeds. Potential plant sources include, but are not limited to, soybean (Kaushik *et al.* 1995), canola (Forster *et al.* 1999; Glencross *et al.* 2003), and lupin (Farhangi and Carter 2001; Glencross *et al.* 2004; Glencross *et al.* 2010). Sources derived from nuts, algae and invertebrates have also delivered promising results (Barrows and Frost 2014). Studies are now showing comparable growth performance measures for rainbow trout (*Oncorhynchus mykiss*) (Barrows and Frost 2014) and Atlantic salmon (Davidson *et al.* 2016) fed fishmeal-free feeds compared with fishmeal inclusive feeds. Even though the amino acid (AA) profiles of some alternative protein sources is favourable for use in aquafeeds, certain factors can affect the nutritional composition of many grains (Glencross *et al.* 2007) and significant problems relating to antinutritional factors (ANF) have been identified in a number of potential plant meal ingredients.

1.3 Functional feeds

Dietary protein is the major and most costly ingredient in compound aquafeed (Wilson 2002). There are 20 amino acids (AA) found in proteins, of which 10, termed essential cannot be synthesized by fish and therefore are required in the diet (Lovell 1998). AA can be subdivided further into those that are used in tissue synthesis and those that have other functions such as being degraded via deamination for energy production, or regulating metabolic pathways crucial to maintenance, reproduction, stress response and immune response (Li *et al.* 2009). See **Table 1.1** (p 6). These are termed functional AA and the supplementation of them, or their biologically active metabolites, is expected to offset some of the adverse effects of using fishmeal substitutes (Li *et al.* 2009).

In recent years advances in aquafeed formulations have led to the development of functional aquafeeds, and environmentally oriented aquafeeds. Functional aquafeeds are supplemented with specific ingredients to enhance health, metabolic transformation, growth performance and/or compositional traits, while environmentally oriented aquafeeds are modified to minimise the negative impacts of environmental factors such as salinity, ammonia, temperature and common husbandry related stressors, on growth and health (Li *et al.* 2009).

Table 1.1 Roles of amino acids in physiological functions and metabolism in fish. Adapted from Li *et al.* (2009). Ala, Alanine; Arg, Arginine; Asp, Asparagine; Cys, Cysteine; Gly, Glycine; Gln, Glutamine; Glu, Glutamate; His, Histidine; Leu, Leucine; Lys, Lysine; Met, Methionine; Phe, Phenylalanine; ser, Serine; Trp, Tryptophan; Tyr, Tyrosine; T3, triiodothyronine; T4, thyroxine

Amino acid	Product	Function	Species	Reference
Amino acids	Various proteins	Structure, transport, regulation, immunity, signaling and fuels	All animals	Li et al., (2007)
Ala, Glu & Ser	Directly	Appetite	Many fishes	Shamushaki et al., (2007)
Arg	Nitric oxide	Kill invaded microorganisms	Channel catfish	Buentello and Gatlin Iii (1999)
Arg	Nitric oxide	Facilitate neurological function and development	Tilapia	Bordieri et al., (2005)
Arg	Nitric oxide	Regulate vascular tone, blood flow, osmolarity in the gill, cell signaling	Killifish	Hyndman et al., (2006)
Arg & Met	Spermine	Induce larval intestinal maturation	Sea bass	Peres et al., (1997)
Arg, Met & Gly	Creatine	High energy storage; antioxidant	Arctic charr	Bystriansky et al., (2007)
Cys, Glu & Gly	Glutathione	Antioxidant & cell signaling	All animals	Wu et al., (2004)
Glu & Gln	Directly	Ammonia removal	Rainbow trout	Anderson et al., (2002)
Glu & Gln	γ-Aminobutyrate	Promote metamorphosis	Abalone	Morse et al., (1979)
Glu & Gln	γ-Aminobutyrate	Regulate food intake	Japanese flounder	Kim et al., (2003)
Glu & Gln	Directly	Increase growth, feed efficiency & gut development	Carp	Yan and Qiu-Zhou (2006)
Gln	Directly	Fuel for macrophage; Cell signaling	Channel catfish	Buentello and Gatlin Iii (1999)
Gln, Gly & Asp	Nucleotides	Genetic information storage and expression, biosynthesis, immunity & reproduction	Various fishes	Li and Gatlin (2006)

Table 1.1 Continued from above. Roles of amino acids in physiological functions and metabolism in fish.

Amino acid	Product	Function	Species	Reference
Gly	Directly	Increase hepatic T4	Rainbow trout	Riley et al., (1996)
His	Directly & carnosine	Protection against pH change	Salmon	Mommsen et al., (1980)
Leu	Hydroxyl-β-methyl-butyrate	Immunity modulation ; Cell signaling	Various fishes	Li and Gatlin (2007)
Lys & Met	Carnitine	Lipid transporter on mitochondrial membrane	Various fishes	Harpaz (2005)
Proline	Hydroxyproline	Enhance growth; Collagen function	Salmon	Aksnes et al., (2008)
Phe & Tyr	T4, T3	Influence metamorphosis	Sole	Pinto et al., (2009)
Phe & Tyr	T4, T3	Enhance growth performance	Channel catfish	Garg (2007)
Phe & Tyr	T4, T3	Influence pigmentation	Japanese flounder	Yoo et al., (2000)
Phe & Tyr	Melanin	Influence pigmentation	Rainbow trout	Boonanuntanasarn et al., (2004)
Phe & Tyr	Epinephrine, norepinephrine	Neurotransmitters that modulate stress response	Flounder	Damasceno-Oliveira et al., (2007)
Trp	Serotonin	Modulate cortisol release, behaviour & feeding	Rainbow trout	Lepage et al., (2002)
Trp	Melatonin	Improve testicular development	Masu salmon	Amano et al., (2004)
Taurine	Directly	Osmotic pressure regulation	Carp	Zhang et al., (2006)
Taurine	Directly	Hardness adaptation	Channel catfish	Buentello and Gatlin (2002)

Stressful husbandry conditions affect AA metabolism in fish (Costas *et al.* 2008; Aragao *et al.* 2010). An impact on total free plasma AA was recorded when Senegalese sole *(Solea senegalensis)* were subjected to confinement, hypoxia, high stocking density or temperature (Costas *et al.* 2008; Costas *et al.* 2012a). Total plasma AA concentrations were higher for rainbow trout and tilapia (*Oreochromis mossambicus*) confined for 24 h (Vijayan *et al.* 1997; Trenzado *et al.* 2003). Other studies have recorded differences in individual plasma AA concentrations, including cases when individual AA concentration differences did not affect the total free plasma AA concentration (Pinto *et al.* 2007; Aragao *et al.* 2008; Costas *et al.* 2012b). Changes for both individual and total plasma free AA concentrations appear to be dependent on the type of stressor, and it is suggested that these changes probably reflect the synthesis of proteins or other compounds related to the stress response (Conceicao *et al.* 2012).

Nutrition has an influence on the health and immune responses in fish (Blazer 1992), and consequently nutritional strategies to modulate fish immune systems and disease resistance appear to be a valuable tool (Conceicao *et al.* 2012). Furthermore the expression of aggressive behaviours has been shown to be reduced through dietary amino acid manipulation in rainbow trout *Oncorhynchus mykiss*, (Winberg *et al.* 2001), orange spotted grouper *Epinephelus coioides* (Hseu *et al.* 2003), and juvenile matrinxa *Brycon amazonicus* (Wolkers *et al.* 2012) .

1.4 Fish Welfare

The rapid expansion in aquaculture output, a consequential reduction in available fish farm sites, the substitution of fish meal and fish oil products in aquafeeds, a trend toward more intensive culture methods are potential threats to welfare. Increased knowledge of the effect of husbandry stressors such as these is driving increased interest in welfare issues for the industry. The maintenance of an environment in which animals can live without fear, hunger or pain, in good health and with all biological systems working appropriately is of ethical and economic importance. There are a wide range of factors that can contribute to the reduced

welfare outcomes of fish in an aquaculture environment, loosely attributed to three interconnected categories: chemical, physical, and social. Chemical refers to the chemistry of their aquatic environment, in particular the presence and concentrations of solid or dissolved nitrogenous waste products, the pH, the saturation of oxygen, carbon dioxide and nitrogen, and the presence of foreign toxins or contaminants. Physical describes the containment structure, as well as associated husbandry and handling procedures such as feeding technique and frequency, fish relocation, grading and slaughter. Social pertains to the development of hierarchies, and exhibited behaviours between individuals. Dawkins (2004) sums up the many welfare indicators with two questions: “Are the animals healthy? And, do they have what they want?” This potentially simplistic approach neither allows for positive experiences, nor the ability for the fish to lead the life of a ‘wild’ counterpart. Paying in-sufficient attention to these factors promotes negative welfare outcomes and provides a stressor for the fish.

A strategy to enhance the welfare of farmed fish is to focus on the production of species that exhibit reduced physiological stress responses to the aquaculture environment (Huntingford and Kadri 2009). Other strategies are more easily applied to species currently farmed, and include efforts to minimise stressors during production husbandry, transport (Iversen *et al.* 2009), harvest / slaughter (Knowles *et al.* 2008; Wilkinson *et al.* 2008), as well as novel feeding technologies (Noble *et al.* 2008) and passive grading systems (Pfeiffer and Freeman 2004). However, there are inherent difficulties in interpreting levels of welfare. Physiological measures such as blood cortisol, glucose and lactate, can be confused by sampling procedure, but can still contribute to an evaluation of welfare. Observations of behaviours such as food intake, food anticipatory behaviour, locomotory activity and ventilation rate are extremely powerful, non-invasive, ‘on-farm’ strategies used to highlight potential welfare problems (Dawkins 2004; Huntingford *et al.* 2006).

1.5 Fish Behaviour

Behaviour is well studied across the animal kingdom, as behaviours define relationships between individuals and thus provide a window into functionality and fitness of populations.

The social behaviour network first described for mammals by Newman (1999) has been shown to be a fundamental and evolutionary conserved feature of the vertebrate brain (Goodson 2005). Studies on a number of species including rats, *Rattus norvergicus*, (Koolhaas *et al.* 1999); pigs, *Sus scrofa (Bolhuis et al. 2003)*; and great tits *Parus major*, (Drent *et al.* 2003) show that the behavioural variation observed within populations is often inherited and that different behavioural phenotypes prosper under different conditions. For example differences in risk taking behaviour between populations of wild vs farmed or hybrid salmon (Einum and Fleming 1997; Johnsson *et al.* 2001; Houde *et al.* 2010)

Displays of aggression are ubiquitous in the animal kingdom, are typically associated with competition for resources such as food, mates, nesting sites or territories, and are viewed as adaptations that can benefit individuals and confer evolutionary fitness. Types of aggressive display have been categorised as anti-predator, defensive, predatory, dominance, maternal, sex-related, territorial and irritable (Moyer 1971; Wilson 1975). More recently a characterisation of offensive (behaviours used in attack) and defensive (not involving an approach to the opponent) has facilitated descriptions of aggressive behaviours across species (Blanchard and Blanchard 1988). However many ethologists consider aggression as part of the more inclusively descriptive 'agonistic behaviour', which refers to any activity related to fighting, whether it be aggression, defence, submission or retreat (Hickman *et al.* 1998).

The impact of agonistic behaviours on growth performance in fish is well researched; behavioural studies on the three-spined stickleback (*Gasterosteus aculeatus*) proliferate and date back to the 1930's (Tinbergen 1951)and a significant amount of work has appeared in the literature since. The manifestations of aggressive behaviour include cannibalism, biting and chasing, and the consequences of these interactions are mortality, damage, disease, and subordination. The effects of the non-fatal consequences are reduced immune function, suppressed feeding, the adoption of inefficient feeding strategies and reduced feed efficiency (Hart 1993). A reduction in food intake is associated with slower growth and a poorer FCR. Thus maintaining chemical, physical and social parameters within an optimal range, is both ethically appropriate and economically sound in a fish farming environment.

In addition to numerous ecological studies, the causes, manifestations and impacts of dominance and aggressive behaviours in aquaculture species have attracted much attention with particular reference to salmonids. Dominant steelhead trout (*Oncorhnychus mykiss*) grew faster, were recipients of fewer aggressive acts, and made better use of tank area than subordinates (Abbott and Dill 1989). Dominant behaviour in Atlantic salmon parr was found to increase with size disparity however the difference in size was considered to be as a result of behavioural competitiveness (described as fierceness), rather than a cause of it (Huntingford *et al.* 1989). In accordance with Huntingford et al. (1989), a further study of Atlantic salmon juveniles at a population level found that differences in levels of aggression were affected most by genetic inter-populational differences (a variation in rates of aggressive encounters between distinct populations), possibly combined with directional selection and domestication effects (a variation in rates of aggressive encounters between second generation (F2) and later generations selected for growth, and wild fish) (Mork *et al.* 1999). The rates of agonistic behaviours of juvenile barramundi (*Lates calcarifer*) held in small groups also proved to differ between discrete populations (Hulse unpublished). Furthermore fish that perform well in a high density tank environment do not always perform well in a low density stream environment (Huntingford and Adams 2005a). Thus different behavioural phenotypes perform best in different environments, with evidence existing that this behavioural variation is inherited (Pottinger and Carrick 2001).

Despite the impacts of agonistic behaviours, and a large body of literature, the neurophysiological basis of aggression is poorly understood in many species (Filby *et al.* 2010). There are many neurological pathways in vertebrates that provide stimulus, both positive and negative, for aggressive behaviour. See Table 1.2 (p 12). The serotonin pathway, remarkably consistent across vertebrate classes, has been shown to play an important role in the modulation of aggressive behaviours in humans (Brown *et al.* 1979), non-human primates (Mehlman *et al.* 1994), mice (Giacalone *et al.* 1968), birds (Sperry *et al.* 2003), reptiles (Deckel 1996; Deckel and Fuqua 1998), and fish (Winberg *et al.* 2001; Hseu *et al.* 2003; Bejo Wolkers *et al.* 2012).

Table 1.2 Neurological pathways of aggression in mammals. Various biochemical pathways have been implicated in either increasing (↑), or decreasing (↓) propensity for aggressive behaviours. Adapted from (Filby *et al.* 2010)

Pathway	Metabolic precursor	Neurotransmitter / hormone	Receptors	Aggression
Dopamine	Tyrosine	Dopamine	Dopamine receptors	↑
Serotonin	Tryptophan	Serotonin	Serotonin receptors	↓
Histamine	Histidine	Histamine	Histamine receptors	↑?
Nitric oxide	L-arginine	Nitric oxide		↑/↓
Somatostatin	Somatostatin I, II, III	Somatostatin I, II, III	Somatostatin receptors	↓
Hypothalamo-neurophysial system	Isotocin	Isotocin	Isotocin receptors	↑/↓
	Arginine vasotocin	Arginine vasotocin	Arginine vasotocin receptors	↑/↓
Hypothalamo-pituitary-interrenal	Hypothalmic factors	Cortisol	Mineral corticoid receptor Glucocorticoid receptor	↓
Hypothalamo-pituitary-gonadal	KISS1	Estradiol, estrone	Estrogen receptor	↑
		Testosterone, 11 ketotestosterone	Androgen receptor	↑

1.6 Tryptophan and the serotonin pathway

Essential amino acids must be provided in the diet in the appropriate concentrations to maximise their use for tissue synthesis, or their availability as precursors in various biochemical pathways. One particular AA of interest is TRP , an essential AA for all animals, required for protein synthesis and subsequent growth, but also implicated in behavioural and physiological stress responses. Protein synthesis typically sequesters approximately half of available TRP (Kalyanasundaram and Ramanamurthy 1983). Optimal dietary inclusion of TRP for barramundi

is approximately 5g.Kg^{-1} of dietary protein (Coloso *et al.* 2004b). This is similar to requirements for other fish and crustaceans, ranging from 3 to 14g.Kg^{-1} of dietary protein (NRC 1992; De Silva and Anderson 1995). Additional TRP up to 13.6 g.Kg^{-1} of dietary protein did not impact on mean weight gain, feed efficiency ratio, or hepatosomatic index of juvenile barramundi over a 12 week period (Coloso *et al.* 2004b). Conversely, dietary inclusion of approximately 2.5 g.Kg^{-1} of dietary protein delivered poorer results for each of the above growth performance parameters.

Tryptophan is one of a group of large neutral amino acids (LNAA) which is transported across the blood brain barrier by a saturable and stereospecific carrier common to the group (Pardridge and Oldendorf 1977). The concentrations of the other LNAA's can affect brain TRP concentration via competition for the carrier site (Johnston and Glanville 1992; Aldegunde *et al.* 1998). In mammals and in fish increasing the level of dietary TRP has a subsequent positive effect on levels of plasma TRP (Culley *et al.* 1963; Johnston *et al.* 1990). An increase in plasma TRP causes an increase in brain TRP (Aldegunde *et al.* 1998; Aldegunde *et al.* 2000) and a subsequent increase in brain serotonin (5-HT) and its primary metabolite 5 hydroxyindoleacetic acid (5OH-IAA) (Johnston *et al.* 1990). In mammals TRP is metabolised via 3 pathways: 1. Hydroxylation and decarboxylation to generate serotonin; 2. deamination and decarboxylation to generate indoleacetic acid; 3. degradation to pyruvate, niacin and acetyl CoA. See Figure 1.1 (p 14). Other important products include NAD, melatonin, and kynurenine.

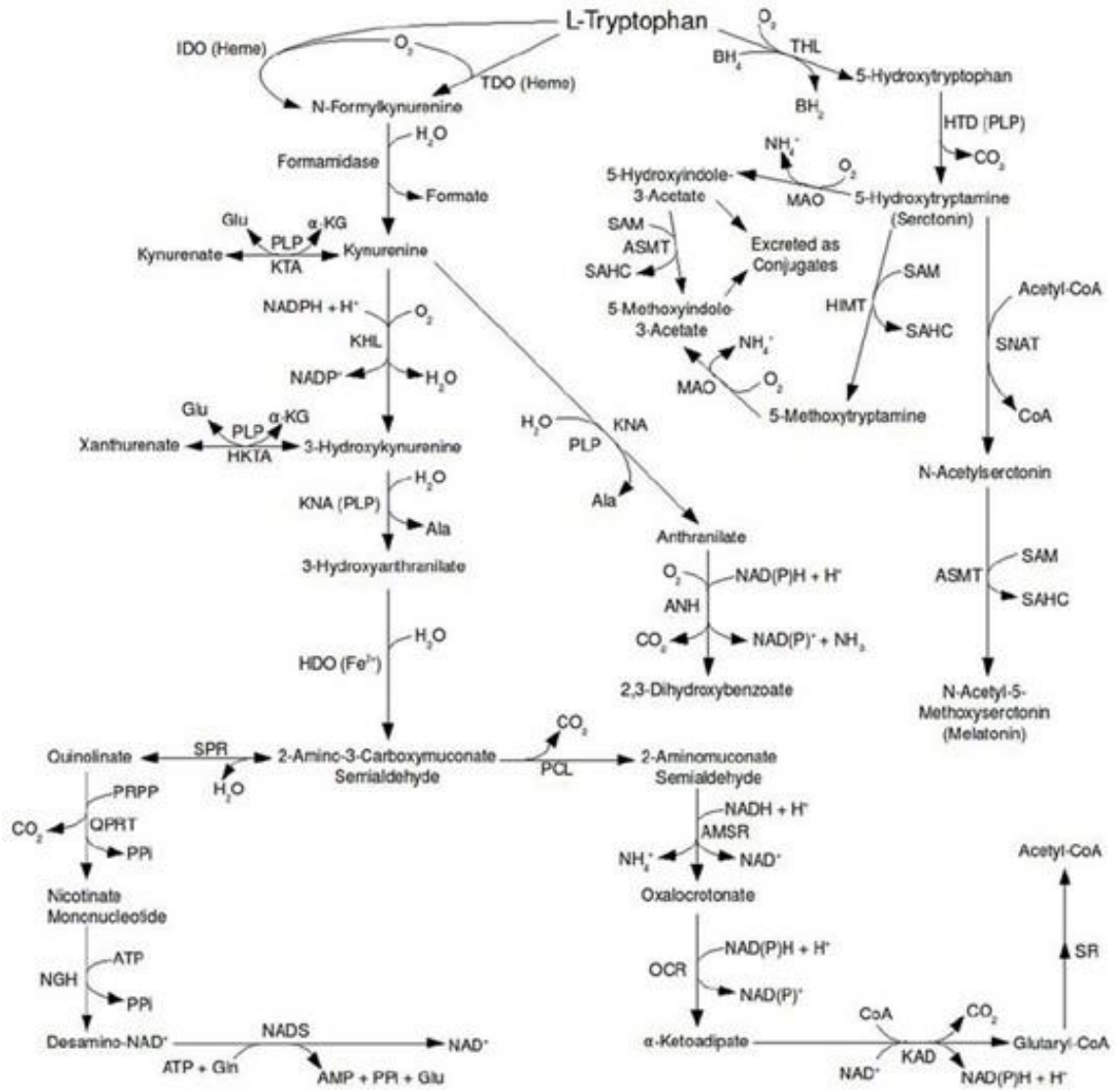

Figure 1.1 Tryptophan catabolism in animals as presented in Yao *et al.* (2011)

The monoaminergic systems of the brain, common to all vertebrates, include the catecholamine neurotransmitters dopamine, epinephrine, and norepinephrine and the indoleamine serotonin (5-hydroxytryptamine, 5-HT). 5-HT is synthesised from TRP. See Figure 1.2 (p 15). TRP is first hydroxylated to 5-hydroxytryptophan (5-HTP) by tryptophan-5-hydroxylase, an enzyme only found in 5-HT synthesizing cells and limited in activity by TRP availability. 5-HTP is decarboxylated by an aromatic L-amino acid decarboxylase to 5-HT.

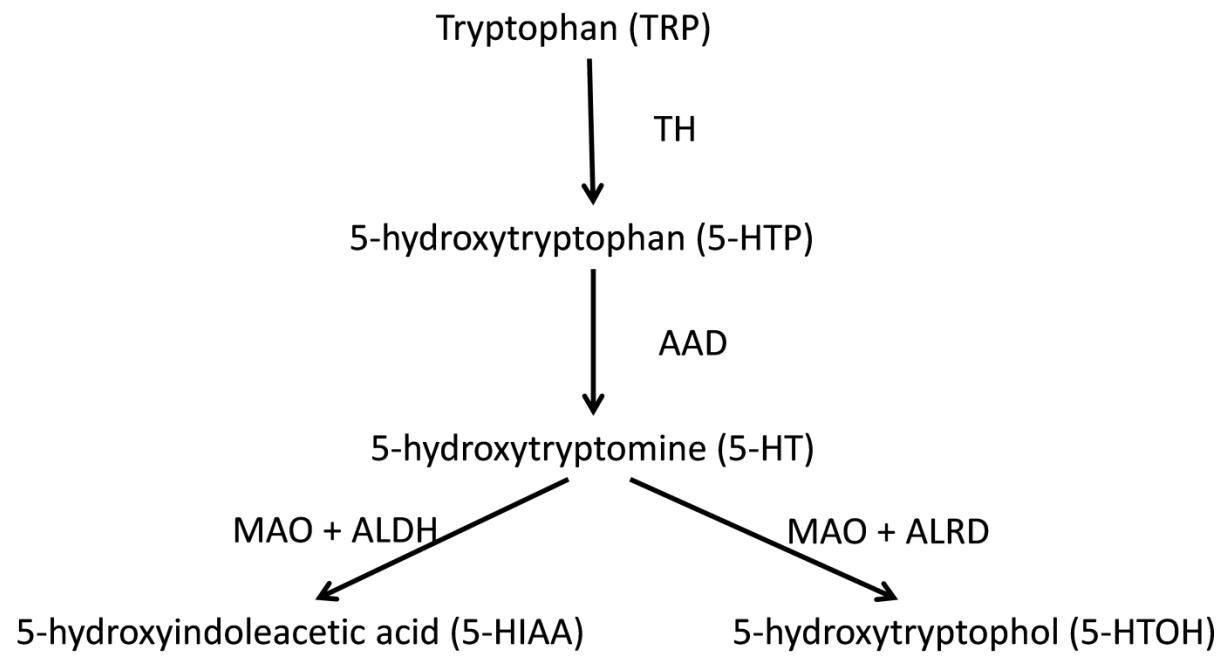

Figure 1.2 Tryptophan metabolism. TH: tryptophan hydroxylase; AAD: aromatic L-amino acid decarboxylase; MAO: monoamine oxidase; ALDH: aldehyde dehydrogenase; ALRD: aldehyde reductase. Adapted from Winberg and Nilsson (1993a).

Brain 5-HT, more than any other neurotransmitter, has the primary neural role in the expression of aggressive behaviours (Nelson and Chiavegatto 2001; Summers *et al.* 2005; Summers and Winberg 2006). Providing supplementary TRP in excess of requirement has been shown to decrease measures of aggression including cannibalism, number of aggressive acts, and latency to attack in a number of fish species. See Table 1.3 (p 17). Despite experiments showing the inhibitory effects on aggression of dietary TRP, an increase in serotonergic activity in the brain has also been demonstrated during aggressive activity (Winberg and Nilsson 1993a), restraint stress (Torres *et al.* 2002), and competition for feed (Cubitt *et al.* 2008). Furthermore, stress coping style and behavioural type have been shown to effect serotonergic

response in a number of species (Winberg and Nilsson 1993a; Nelson and Chiavegatto 2001; Koolhaas *et al.* 2007; Winberg and Thörnqvist 2016), as has time of emergence of salmon alevins from the redd with earlier emerging fish being bolder and more aggressive (Thornqvist *et al.* 2015).

Table 1.3 Impacts of supplementary dietary tryptophan on aggressive behaviours, stress physiology, growth, feeding and nutrition in a range of teleost fish. Table adapted from Conceicao *et al.* (2012). ↑ Indicates and increase; ↓ indicates a decrease; → indicates no change. LNAA large neutral amino acids

Welfare issue	Species	Feed TRP conc. (g/Kg)	Duration (days)	Results	Reference
Aggression	Rainbow trout Oncorhynchus mykiss	8.38 & 74.30	3 and 7	↓ Number of aggressive acts (7 days); → attack latency	Winberg et al. (2001)
	Atlantic cod *Gadus morhua*	27.6 & 28.1	7	↓ Aggressive acts ↑ Latency to first aggressive act	Hoglund et al. (2005)
	Grouper *Epinephelus coioides*	2.5, 5.0, 10	10	↓ Cannibalism	Hseu et al. (2003)
	Fighting fish *Betta splendens*	2.8 & 26.6	7	→ Duration of opercula display toward mirror	Clotfelter et al. (2007)
	Mud crab *Scylla serrata*	3.2, 5, 7.5, 10	15 and 30	↓ Aggression	Leopoldo et al. (2010)
	Matrinxa *Brycon amazonicus*	9.4, 18.8, 37.6	7	↓ Aggression (9.4g/kg)	Bejo Wolkers et al. (2012)

Table 1.3 Continued. Impacts of supplementary dietary tryptophan on aggressive behaviours, stress physiology, growth, feeding and nutrition in a range of teleost fish. Table adapted from Conceicao *et al.* (2012). ↑ Indicates and increase; ↓ indicates a decrease; → indicates no change. LNAA large neutral amino acids

Welfare issue	Species	Feed TRP conc. (g/Kg)	Duration (days)	Results	Reference
Stress response (cortisol)	Rainbow trout Oncorhynchus mykiss	8.38 & 74.3	3 and 7	→ Plasma cortisol	Winberg et al. (2001)
		0.44, 0.95, 1.84, 3.57	7	↑ Basal plasma cortisol in non-stressed fish ↓ Plasma cortisol in stressed fish	Lepage et al. (2002)
		3.57	3, 7, and 28	↓ Plasma cortisol in stressed fish (7 days)	Lepage *et al.* (2003); Lepage *et al.* (2005)
	European seabass Dicentrarchus labrax	5	7	↓ Plasma cortisol in stressed fish	Herrero et al. (2007)
	Matrinxa *Brycon amazonicus*	9.4, 18.8, 37.6	7	→ Plasma cortisol	Bejo Wolkers et al. (2012)

Table 1.3 Continued. Impacts of supplementary dietary tryptophan on aggressive behaviours, stress physiology, growth, feeding and nutrition in a range of teleost fish. Table adapted from Conceicao *et al.* (2012). ↑ Indicates and increase; ↓ indicates a decrease; → indicates no change. LNAA large neutral amino acids

Welfare issue	Species	Feed TRP conc. (g/Kg)	Duration (days)	Results	Reference
Growth, feed intake and efficiency, and survival	Rainbow trout Oncorhynchus mykiss	8.38 & 74.3	3 and 7	→ Feed intake	Winberg et al. (2001)
		0.44, 0.95, 1.84, 3.57	7	→ Feed intake	Lepage et al. (2002)
		3.57	3, 7, and 28	→ Feed intake	Lepage *et al.* (2003); Lepage *et al.* (2005)
		26.9	77	↓ Growth (SGR); ↑ Feed efficiency (FCR); ↑ Feed intake	Papoutsoglou et al. (2005)
		3.9 & 5.9	98	→ Growth; → Feed intake	Johnston et al. (1990)
	Barramundi *Lates calcarifer*	1.1, 2.1, 3.1, 4.1, 5.1, 6.1	84	↓ Growth (SGR) and feed efficiency (FER) at 1.1g/kg inclusion	Coloso et al. (2004)
	Grouper *Epinephelus coioides*	2.5, 5.0, 10	10	↓ Growth	Hseu et al. (2003)
	Brown trout *Salmo trutta*	TRP:LNAA of 0.22	7	↑ Feed intake recovery post stress	Hoglund et al. (2007)
	Mud crab *Scylla serrata*	3.2, 5, 7.5, 10	15 and 30	↑ Survival; ↓ Growth	Leopoldo et al. (2010)

1.7 Stress physiology

Stress is a loosely used term and even within biological contexts stress, stressors and stress responses have relatively non-finite boundaries in their description. The physiological stress response involves a series of biochemical and physiological changes in an attempt to compensate for the challenge, and thereby cope with the stress (Wendelaar Bonga 1997). Stressors in teleost fish are described as producing a set of coordinated behavioural and physiological responses, which are categorized in numerous ways (Wendelaar Bonga 1997). Disruption to homeostasis delivers what has been categorized as a three tier response: primary, a neuroendocrine release of catecholamines and corticosteroids along with fight or flight; secondary, physiological changes including increase of red blood cells and elevation of circulating glucose and behavioural changes such as colouration or reduced feeding, and tertiary, long-term whole animal responses such as depressed growth and reproductive performance, and elevated occurrence of disease and mortality (Ellis *et al.* 2012). Persistent behavioural responses such as subordination or reduced food intake, while reliable indicators of underlying conditions, are most likely mediated via a specific or integrated endocrine response. Furthermore, evidence suggests that stress and aggressive behaviours are linked as they both mobilise activity in specific brain regions (Summers and Winberg 2006).

Elevated cortisol is implicated in numerous conditions of concern to commercial aquaculture: feeding depression, reduced locomotor activity, modified shoaling / schooling, poorer FCR's, greater size variance, reduced reproductive performance and increased incidence of disease and mortality (Ellis *et al.* 2012). The effects of agonistic behaviour, such as the formation of dominance hierarchies, include elevated stress responses, and are thought to be responsible for considerable production losses in many fish culture systems (Winberg and Nilsson 1993a). However, hypothalamic corticotropin releasing hormone (CRH) activation and subsequent cortisol production should not necessarily be adversely viewed; during smoltification benefits are conferred for Atlantic salmon from elevated cortisol, as treatment with cortisol prior to SWT enhances hypo-osmoregulatory capacity in salmonids (Madsen 1990; Fuentes *et al.* 1996). Furthermore, unsuccessful transfers of salmon to saltwater have resulted in the transfer-

associated cortisol peak being maintained, probably as a result of failed homeostatic mechanisms (Franklin *et al.* 1992).

Hypothalamic pituitary interrenal (HPI) axis activation has been linked with both catecholaminergic and serotonergic activity in salmonids (Winberg and Nilsson 1993a). Administration of TRP supplemented feed delivered an increased cortisol response in unstressed fish and a reduced cortisol response in stressed fish (Lepage *et al.* 2002). Acute stressors administered to rainbow trout for as little as 15 seconds have been shown to immediately elevate brain 5HT:5HIAA and for the effect to remain for 4 hours (Gesto *et al.* 2013). During chronic stress, plasma cortisol levels have been observed to return to resting levels despite continued exposure to the stressor (Vijayan and Leatherland 1990).

1.8 Summary and aims

Welfare impacts of the culture environment, including but not limited to factors such as dietary ingredients, feeding regimes, ration size, feeding hierarchies, dominance hierarchies, stocking densities, novel environments, and salinity change, on stress physiology and behavioural responses are evident. Species-specific behaviours, such as cannibalism in juvenile barramundi, also compromise welfare, both directly via mortality, and indirectly via injury, increased available pathogen sites, and handling stressors associated with prevention strategies such as size grading. Rates of cannibalism in the early stages of culture range typically from 20% to 50% of the cohort. A wasting condition, termed *pin-heading* or *failed smolt syndrome*, apparently associated with the transfer of Atlantic salmon smolts from fresh to seawater, describes the cessation of feeding and subsequent loss of condition of some fish at marine transfer, and has significant impacts on productivity, as well as welfare implications, with reports of up to 30% of smolts failing to resume feeding post-transfer. Despite the large mortality observed during the early phases of barramundi culture and the marine transfer phase for Atlantic salmon, research into any positive effects of dietary AA manipulation remain unpublished. The varied biochemical utilization of various AA, particularly TRP, suggests the use of supplementary dietary TRP may, via the serotonin pathway and its subsequent effects on stress physiology and

aggressive behaviours, be useful in mitigating stock losses and in increased welfare outcomes for both barramundi and Atlantic salmon. To improve production and welfare outcomes for the commercially important species barramundi and Atlantic salmon, this work examines three broad questions:

- Dietary TRP reduces aggressive behaviour in aggressive individuals and suppresses hunger. Aggressive behaviour and domination of the food supply are primary factors in growth depensation in fish populations. Intracohort cannibalism by barramundi can only occur when size variability is present. Therefore will the supplementation of fish food with TRP reduce the occurrence of aggressive interactions, and prevent individuals from dominating the food supply, with the effect of reduced growth depensation and therefore reduced cannibalism? (Chapter 2, Exp 1 and Chapter 3 Exp 3 & 4)
    - Hypothesis: Supplementing fish food with TRP will reduce the occurrence of cannibalism by juvenile barramundi, compared to those fed a non-TRP supplemented feed

- Aggressive behaviour and cannibalism is common between barramundi. Are some fish more aggressive than others and can they be identified by consistent responses to stimuli, and by stress and serotonergic response to an intruder fish? (Chapter 2, Exp 2)
    - Hypothesis: When presented with 3 different stimuli, juvenile barramundi will respond consistently in behaviour, endocrine stress and serotonergic response depending on their behavioural type

- Dietary TRP moderates the physiological stress response (plasma cortisol) in stressed fish. Seawater transfer of Atlantic salmon smolt causes an acute and sustained cortisol response. A severe reduction in feeding at this stage is attributed to physiological stress

response. Therefore will supplementation of fish food with TRP moderate the physiological stress response, and result in higher food intake? (Chapter 4)

- Hypothesis: Supplementing fish food with TRP will increase food intake in Atlantic salmon smolt at SWT via moderation of HPI axis response compared to those fed a non-TRP supplemented feed

This thesis will focus on the behavioural, biochemical and stress physiology responses of barramundi (Chapter 2, p 25 & Chapter 3, p 77) and Atlantic salmon (Chapter 4, p 131) when exposed to common culture stressors after being fed diets with supplementary TRP. The main objectives of the study for barramundi are to quantify aggressive behaviours, baseline biochemical responses associated with the serotonin pathway, and baseline physiological stress responses, and any associated impacts of the treatment diets. In addition the study will address growth performance and growth depensation. The main objectives of the study for Atlantic salmon are to assess the impact of TRP supplemented diets on resumption of feeding of smolt post-marine transfer, biochemical responses associated with the serotonin pathway, and stress physiology.

Specifically this research will examine whether:

- Supplementary dietary TRP effects the behavioural response of a resident barramundi to a 50% smaller conspecific;
- Behavioural responses by juvenile barramundi are predictable based on previous responses to a mirror image, a foreign object, and a 30 % smaller conspecific intruder;
- Physiological stress response and brain serotonergic activity reflects behavioural type and / or recent behavioural response to a 30% smaller conspecific intruder;
- Supplementary dietary TRP affects food intake, FCR, growth rate and growth depensation of juvenile barramundi over a 50 day period;
- Supplementary dietary TRP affects rates of cannibalistic-associated mortality in juvenile barramundi over a 50 day period;

- Ration size affects rates of cannibalistic-associated mortality in juvenile barramundi over a 50 day period;

- Supplementary dietary TRP affects the physiological stress response of barramundi or Atlantic salmon, as indicated by blood cortisol, glucose and lactate concentrations and by whole body (barramundi) cortisol concentrations;

- Supplementary dietary TRP effects the physiological stress response, as indicated by serum cortisol concentrations, in Atlantic salmon smolts at SWT and whether the physiological stress response at 24 h post-transfer is affected by transfer type, ie. high stress or low stress;

- Feeding depression commonly observed in Atlantic salmon smolts at SWT can be attributed to transfer type, ie. high stress or low stress;

- Supplementary dietary TRP moderates the feeding depression observed in Atlantic salmon smolts at SWT.

2 Chapter 2 - Aggressive behaviours of juvenile barramundi exposed to a range of provocative stimuli and their associated brain serotonergic and physiological stress responses

2.1 Introduction

The behaviour of fish in an aquaculture setting is a primary welfare indicator, and thus of utmost commercial importance. Behavioural responses to stressors are not only stressor specific but also depend upon the coping style of the individual (Koolhaas *et al.* 1999). In the natural environment fish may be solitary or sociable, however in an aquaculture environment group living at high density is the "norm", and though group living in the wild confers numerous benefits, competition for resources and territory within a tank or cage can lead to the emergence of competitive behaviours (Jobling and Koskela 1996). Consequently the study of fish behaviour, especially species from the commercially important salmonid group, has been well researched.

Behavioural responses have been shown in rainbow trout (*Oncorhynchus mykiss*); see review by Ellis *et al.* (Ellis *et al.*), and Atlantic salmon (*Salmo salar*) to be situation specific, where for example fish that monopolized the food supply in a tank were unable to secure feeding stations and showed very low levels of aggression in a stream setting (Huntingford and Adams 2005b). Despite these results there is considerable evidence for behavioural syndromes, such as an aggressive syndrome, where some animals are more aggressive whereas others are less aggressive across a range of situations and contexts (Sih *et al.* 2004), much like humans show consistent individual differences in specific behaviours (Pervin and John 1991). In an aquaculture environment behavioural syndromes are important, because it is the manifestation of behavioural type, within syndrome, that strongly predicts the ability of individuals to maintain their feeding, or hierarchy rank order within the population.

Dominant or subordinate status has been investigated in fish by pairwise interactions. Placing a pair of fish together in a confined environment will trigger agonistic activity that results in the establishment of a dominant-subordinate relationship (Johnsson *et al.* 1996; Overli *et al.* 1999;

Pottinger and Carrick 2001), while in territorial species agonistic behaviour increases with incursions by other individuals.

Barramundi are ambush predators and Type 2 cannibals; they consume their prey whole and head first. To achieve this the mouth of the predator must be of equal or greater size than the body depth of the prey. In barramundi a relationship between fish length and mouth size exists, and thus the capacity for cannibalism can be described by differences in total length. A predator can consume a prey up to 70% of its own length; however a preference is displayed for smaller individuals. Whilst alternate endpoints were considered in light of animal welfare and ethics approval, it was considered necessary to use realistic situations. To address possible differences in response to differing sized intruders, whilst still allowing for the possibility of cannibalism, intruders of 50% and 70% the length of the resident fish were used in the current study. With the level of aggressive response to a specific stimulus, such as their own mirror image, in an experimental setting has fewer welfare implications than in dyadic studies, and allows individuals to be placed on a spectrum. The ability to identify behavioural type reveals more about how individuals may respond to increased serotonergic activity.

The response of fish to their own reflection is often aggressive suggesting they do not possess self-recognition (Tinbergen 1951). Therefore, when a fish responds to its reflection, it is responding to what it believes is a conspecific of its exact same size. African cichlid fish *Astatotilapia burtoni* exposed to either a mirror image or a conspecific, displayed no differences in plasma testosterone or 11-ketotestosterone , which were drastically elevated compared to controls (Desjardins and Fernald 2010). Responses of fish to foreign or novel objects appear to differ between species,individuals, andcontext. A shy-bold continuum exists for Pumpkinseed sunfish *Lepomis gibbosus* and responses are consistent across time though not across context, meaning that individuals respond consistently to specific stimuli however responses vary between stimuli (Wilson *et al.* 1993; Coleman and Wilson 1998). A similar thermopreferendum is reported in zebrafish (Rey *et al.* 2015) . Bold, risk takers have a preference for warmer water while shy, less risk-prone individuals favour cooler water. Considering the appeal of barramundi as a species for commercial production the scientific

literature is relatively limited and predominately favours nutritional studies. Some work has been carried out on cannibalism amongst barramundi with the focus on size and morphometrics (Parazo *et al.* 1991; Ribeiro and Qin 2013; Ribeiro *et al.* 2015) and mitigation strategies (Qin *et al.* 2004; Appelbaum and Arockiaraj 2010). The effects of intracohort aggressive behaviour amongst juvenile barramundi include increased growth depensation and thus greater opportunity for cannibalism to occur, and therefore the reduction in agonistic behaviour and the identification of aggressive individuals provide valuable research questions.

Larval rearing technology and bio-security measures have improved significantly over the past 2 decades. Despite these advances, typical larval barramundi survival rates through metamorphosis in industry consistently range from 50% to 80% (Gore, personal communication, Darwin Aquaculture Centre), and while mortality may be partially ascribed to failure to wean from live feeds to artificial diets (Bosmans *et al.* 2004), it is predominately as a result of cannibalism (Parazo *et al.* 1991). Differences in the genetic background of individuals and variable growth between fish contribute to this high occurrence of aggressive interactions and cannibalism in the barramundi industry (Qin *et al.* 2004). To combat this agonistic behaviour, frequent management intervention (i.e. size-grading of individuals) is required. This labour-intensive process starts at metamorphosis, when juveniles are capable of surviving such a stressful operation, and continues up to 3 times per week throughout the nursery phase (Harrison, personal communication, Mainstream Aquaculture). Frequent grading of barramundi between metamorphosis and approximately 100 mm is viewed by industry as a necessary activity to reduce mortalities associated with cannibalism. These grading operations are costly, use large amounts of water in recirculating systems, and are stressful for the fish. Outbreaks of disease can often be linked back to poor husbandry during grading operations (Ashley 2007).

Given the deleterious effects of aggressive interactions between barramundi on both welfare and production cost measures, experiments were designed to answer the questions: Does supplementary dietary TRP moderate the response of barramundi toward potential conspecific prey? Finally, are behavioural responses predictable in barramundi based on response to stimuli, serotonergic and physiological stress response? The following hypotheses were tested:

- Hypothesis: Supplementing fish food with TRP will reduce the occurrence of cannibalism by juvenile barramundi, compared to those fed a non-TRP supplemented feed

- Hypothesis: When presented with 3 different stimuli, juvenile barramundi will respond consistently in behaviour, endocrine stress and serotonergic response depending on their behavioural type

Specifically these studies aim to:

- Quantify the aggressive behavioural response of a resident barramundi to a conspecific 50% smaller in size;
- Examine whether supplementary dietary TRP effects the behavioural response of a resident barramundi to a 50% smaller conspecific;
- Quantify the response of juvenile barramundi to a mirror image, a foreign object, and a 30 % smaller conspecific intruder, and examine whether behavioural responses are predictable based on previous responses and physiological stress response or brain serotonergic activity.

2.2 Materials & Methods

Two experiments were conducted. Firstly, to examine whether supplementary dietary TRP altered the behavioural response of a resident fish toward a 50% smaller intruder. Secondly, to assess behavioural responses of juvenile barramundi to mirror images and foreign objects and whether these responses were consistent with responses toward an intruder conspecific.

2.2.1 Experiment 1 – Response to intruder

2.2.1.1 Fish and experimental system

Juvenile barramundi in two size groups, 20-25 mm and 40-50 mm hereafter referred to as intruders and residents respectively, supplied by WBA Hatcheries (West Beach, South Australia), were air freighted to Launceston airport and transported by road to the Aquaculture Centre tropical facility, IMAS Launceston (University of Tasmania), Australia (41^0S) in February 2012. On arrival resident fish were anaesthetised in iso-eugenol (18.75mg.L^{-1} AQUI-S, New Zealand) weighed, measured and randomly allocated to chambers (Figure 1) in one of two identical experimental systems for acclimation (1 fish [0.79 ± 0.006 g; 41.3 ± 0.08 mm] per chamber; initial stocking density 0.105 kg.m^{-3}). Excess residents were housed en masse in an 80 L holding tank, as were all the intruders in another 80 L holding tank. Both experimental and holding systems used recirculating technology and were supplied with freshwater at 30^0C (by immersion heater) at a rate of 0.6 and 5.4 l.min^{-1} respectively, and a 24-h light: 0-h dark photoperiod (Barlow *et al.* 1995).

Two separate experimental recirculation systems (i.e. one for the reference feed and one for the feed with additional TRP) were used to eliminate the possibility of water-borne TRP contamination, comprising 3 replicate (per feed) glass aquaria (120 cm x 42 cm x 40 cm) divided with white PVC walls into 20 chambers (12 cm x 40 cm x 20cm) of 7.5 L each. Each of the three replicated aquaria were longitudinally identified by alphanumeric labelling, each aquarium containing two rows of 10 chambers, with each row being a time (day) treatment of 10 replicates. The inside walls of each chamber were fitted with white corrugated plastic inserts to eliminate disturbance during observation and reflection. Six overhead cameras (C500 CCD,

Swann) were fitted to record behavioural interactions for 5 minutes post-introduction of the intruder and for 5 minutes in each hour for the following 24 hours. Recordings were made after 1, 2, 3, 4, 5, 6, 7, 8, and 14 days delivery of the prepared feeds. Each chamber had its own water inlet, while a central drain running the length of each aquarium circulated water through the filtration system. The experimental systems were supported by particulate filtration (dacron screen) and a 60 L submerged bead filter. See Figure 2.1 (p 30). Dissolved oxygen was maintained at >80% saturation by aeration and water quality (ammonia, nitrate and nitrate) was monitored daily. Water exchanges were performed as necessary to maintain water chemistry within the appropriate range for the species (Tucker *et al.* 2002).

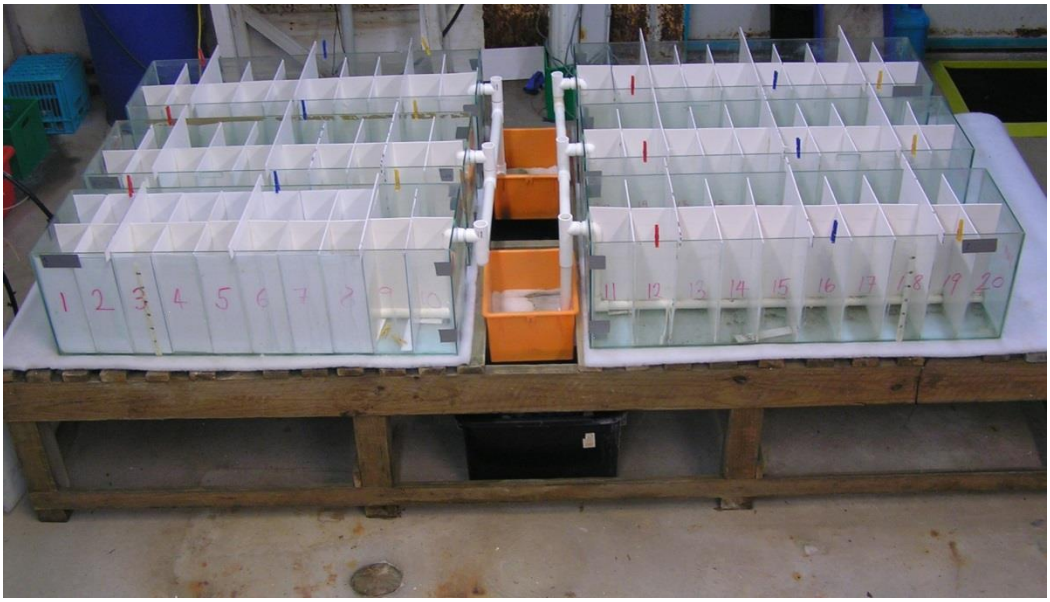

Figure 2.1 Experimental system comprising 60 x 7.5L compartments per feed showing separate particulate filter (orange tub) and sump / biofilter (black tub) for TRP supplemented and control feeds.

On the 17th of February 2012 resident fish were removed from row A (1 day) chambers and re-measured (1.52 ± 0.015 g; 47.8 ± 0.24 mm) to provide accurate data from which to select intruders half (50.21 mm ± 0.004 % and 50.36 mm ± 0.0034 % respectively for TRP and control feeds) their size. The following morning intruder fish were anaesthetised, weighed, measured,

recovered from anaesthesia and when fully responsive, added to the chambers containing the larger resident fish. This process was repeated for each of the day treatments with days 7, 8 and 14 re-using rows A, B and C after the conclusion of the 1, 2, and 3 day studies. At the conclusion of each study fish were weighed, measured and intruder survival was recorded, following which fish were returned to holding tanks

2.2.1.2 Experimental feed

Two semi-purified, isonitrogenous and isoenergetic feeds using recipes (See Table 2.1, p 32) modified from Katersky and Carter (2005), were made comprising a reference feed (Feed 1) similar to a commercial feed appropriate for barramundi at this stage of development and a feed with supplementary TRP (Feed 2). Fish meal, fish oil, vitamin and mineral premix and TRP was supplied by Ridley Aquafeed (Narangba, QLD, Australia). α-cellulose replaced supplementary crystalline TRP in the control feed. Pellets of 2 mm diameter were manufactured using a pasta maker, dried over 48 h at 30°C and stored at -20°C until ready to use. Fish were fed an individually pre-weighed ration of 8% of body weight per day (% BW/day) based on industry recommendation for fish of this size, in a single ration (09.00 h) so as to show behaviour in relation to a single daily dose and to reduce feed anticipatory behaviours. Feed anticipatory behaviour, the effect of increased activity prior to regular mealtimes is common especially if fish are delivered a below satiation ration. In the current study, the presence of a single dominant fish and the relatively short time of the study, it is expected that feed anticipatory behaviour was minimized. A feed conversion ratio (FCR) during the acclimation period was calculated by dividing wet weight gain by food intake, and used to predetermine daily ration increases. All feed was consumed. During acclimation to the system all fish were fed with Feed 1. The transfer of fish to a novel environment invariably triggers a depressed feeding response which can last for two weeks. Fish were considered acclimated when the full ration was consumed within 2 minutes without waste.

Table 2.1 Composition of reference and TRP supplemented food fed to barramundi over the duration of the experiment. Feeds were analysed for proximate composition via kjeldahl and for amino acid composition via HPLC.

Ingredient composition (mg.g^{-1}) DM	**Ctrl**	**TRP**
Fishmeal	789.00	789.00
α-cellulose	28.00	14.00
Wheat gluten	76.00	76.00
Vitamin and mineral premix	20.00	20.00
CMC	10.00	10.00
Stay C	20.00	20.00
Fish Oil	36.95	36.95
Phosphorous	10.00	10.00
Choline chloride	10.01	10.00
Tryptophan	0.00	14.00
TOTAL (g)	999.96	999.95
Water (ml.kg^{-1})	389.66	389.63
Pellet size (mm)		
2mm	8.29	10.13
Liquid hydrolysis + UPLC (mg.g^{-1}) DM		
Tryptophan	7.23	19.40
Chemical composition (%)		
Crude protein (%)	62.2%	61.0%
Crude lipid (%)	11.1%	11.5%
Moisture (%)	7.7%	8.2%
Ash content (%)	6.4%	6.4%

2.2.1.3 Behavioural analysis

Six overhead cameras (C500 CCD, Swann) recorded behavioural interactions after 1, 2, 3, 4, 5, 6, 7, 8, and 14 days of ration, for 5 minutes post introduction of the intruder and for 5 minutes in each hour for the following 24 hours. Footage was recorded and on playback seven behavioural actions were scored. Four aggressive acts were defined as: (1) Chasing: Resident approaches intruder, elicits flight response, and pursues for more than 3 intruder body lengths; (2) Eyeballing: Resident orients toward intruder within 1 resident body length (It is acknowledged

that eyeballing is not a term commonly used within the fish behaviour literature. It is particularly pertinent to barramundi behaviour. Barramundi are ambush predators, and in confinement with potential prey exhibit a stalking behaviour that involves a level of observation greater than inspection. It is a level of observation that always pre-empts a cannibalistic strike and thus I feel is a justified description in line with the dictionary definition: 'eyeballing' is: To look at, check, or observe closely, *two opponents eyeballing each other*.; (3) Attack: Resident makes failed attempt to grab intruder; and (4) Grab: Resident grabs intruder head-first. One non aggressive act defined as: (5) Ignore: Resident is neither chasing nor eyeballing intruder; and two behavioural states exhibited in conjunction with described behaviours, defined as: (6) Head shaking: Resident exhibits head shaking behaviour; and (7) Fast opercula: Resident exhibits rapid and exaggerated opercula movement,. Latency to act, described as the duration between the start of each 5 minute observation period and the display of a listed behaviour, number of acts, bout length, and act sequence were scored using JWatcher (Blumstein *et al.* 2006). Behaviours were analysed across three timescales: Initial confinement, up to 6 h post introduction, and up to 24 h post introduction.

2.2.1.4 Initial confinement

Total aggressive acts (combination of behaviours 1-4), total time spent chasing, attack occurrence, and latency to eyeballing and chasing of a larger resident barramundi toward a 50% smaller conspecific were examined from video recordings of the first 5 minutes of confinement on days 1, 2, 4, 6 and 14 of fish fed either reference or TRP supplemented food. Data are derived from 9 replicates per feed type.

2.2.1.5 Up to 6 h post-introduction

Mean aggressive acts (combination of behaviours 1-4, mean occurrence of attacks, mean time spent chasing (mean of 9 replicates per feed across each 5 minute period), average chase duration (mean from each 5 minute period averaged across 9 replicates per feed), and latency to eyeballing and chasing of a larger resident barramundi toward a 50% smaller conspecific were examined over the first 6 hours of confinement. Behaviours were scored from video

recording 5 minutes at introduction and 5 minutes of each hour for the first 6 hours on days 1, 2, 4, 6 and 14 of fish fed either reference or TRP supplemented food. Data are derived from 9 replicates per feed type. Survival between feed types and trends in survival across time were examined.

2.2.1.6 Up to 24 h post introduction

Mean time spent chasing (without other behaviours) per 5 minutes, and mean time spent chasing (including whilst headshaking (HS) and with fast opercula (FO)), mean aggressive acts (combination of behaviours 1-4) and mean occurrence of attacks per 5 minutes by a larger resident barramundi towards a 50% smaller intruder conspecific were examined over the first 24 hours of confinement. Behaviours were scored from video recordings of 5 minutes at introduction and 5 minutes of each hour for 24 hours, after both 1 and 14 days of either reference or TRP supplemented food. Data are derived from 9 replicates per feed type. Distributions were square root transformed for normality. Survival between feed types and trends in survival across time were examined.

2.3 Materials & Methods

2.3.1 Experiment 2 – Behavioural responses to 3 stimuli

2.3.1.1 Fish and experimental system

Forty resident fish were individually exposed to three separate stimuli for one hour each whilst isolated in the previously described chambers. Described behaviours were quantified as was the orientation of the resident. Three days of acclimation preceded exposure to each stimulus .The initial test assessed the behavioural reaction of these fish to their own reflection in a pre-positioned but obscured mirror (cover removed during the one hour test). The second test assessed their response to the introduction of a foreign object (FO) to the chamber, and the third and final test examined their responses toward a smaller conspecific intruder. Biological samples for the measurement of physiological stress responses and brain serotonergic activity resulting from exposure to a smaller conspecific (the third test) were taken on completion of the test.

Juvenile barramundi in two size groups, 50-55 mm and 60-65 mm hereafter referred to as intruders and residents respectively, supplied by WBA Hatcheries (West Beach, South Australia), were air freighted to Launceston airport and transported by road to the Aquaculture Centre tropical facility, IMAS Launceston (University of Tasmania), Australia (41^0S) in April 2013. On arrival resident fish were anaesthetised in iso-eugenol (18.75mg.L^{-1} AQUI-S, New Zealand) weighed, measured and randomly allocated to 40 chambers in one of two identical experimental systems (as previously described) for acclimation 1 fish (mean 2.21 ± 0.04 g; 60.6 ± 0.07 mm) per chamber at an initial stocking density of 0.294 kg.m^{-3}. Forty intruders (mean 1.77 ± 0.008 g; 52.3 ± 0.06 mm; stocking density 0.236Kg.m^{-3}) underwent the same process and were housed in identical but separate chambers. Excess residents were housed en masse in an 80 L holding tank, as were all the intruders in another 80 L holding tank. Both experimental and holding systems used recirculating technology and were supplied with freshwater at 30^0C (by immersion heater) at a rate of 0.6 and 5.4 l.min^{-1} respectively, and a 24-h light: 0-h dark photoperiod (Barlow *et al.* 1995).

On the 10th April 2013, 3 days prior to the first stimulus test (mirror image) resident fish were removed from chambers and re-measured (mean 3.87 ± 0.029 g; 67.9 ± 0.24 mm: stocking density 0.516 $Kg.m^{-3}$) and this was considered the start of the experiment. A recovery period of 3 days was provided between tests and thus the foreign object test was conducted on the 16th April 2013 and the intruder test on the 19th April 2013. The day preceding the intruder test, residents and intruders were anaesthetised in iso-eugenol (18.75$mg.L^{-1}$ AQUI-S, New Zealand), weighed and measured to provide accurate data from which to select intruders (2.47 ± 0.031 g; 59.18 ± 0.22 mm) of approximately 70% the length of the residents (7.48 ± 0.039 g; 82.43 ± 0.3 mm; stocking density 0.997 $Kg.m^{-3}$). Average stocking density during the intruder test was 1.33 ± 0.008 $Kg.m^{-3}$. Immediately following the intruder test resident fish were removed from the chambers, anaesthetised, sampled with handheld devices for blood glucose (ACCU-CHEK Performa Nano, Roche Diagnostics, Germany), and lactate (Lactate Pro, ARKRAY Inc. Japan), prior to euthanasia via severance of the spinal cord. The brain was then expeditiously removed by opening the cranium with a downward scalpel incision toward the mouth and immersed in liquid nitrogen. The removal and freezing of the brain took less than 90 seconds. Brains were transferred to 80°C storage.

2.3.1.2 Experimental feed

Fish were fed a pelleted feed identical to the reference feed delivered in experiment 1 (Table 1). A daily ration of 8% of body weight was delivered equally over two meals between 09.00h - 10.00h and 16.00-17.00h. All feed was consumed.

2.3.1.3 Calculations

Feed conversion ratio (FCR) was calculated as:

$$FCR\ (g.g^{-1}) = \text{Weight of ingested feed (g)} / \text{Weight gain (g)}$$

Specific growth rate (SGR) was calculated as:

$$SGR\ (\%\ d^{-1}) = 100\ ((\ln W_t - \ln W_i)/t)$$

where W_i and W_t are initial and final weights respectively and t is time (days) between initial and final weighing.

The coefficient of variation (CV) was calculated as:

$$CV = 100\ (\text{standard deviation} / \text{mean})$$

2.3.2 Statistics

Statistical analyses were performed using SPSS version 21 (SPSS, 2014) and GraphPad Prism version 6.0 for Windows, GraphPad Software, La Jolla California USA. Mean values are reported ± standard error of the mean (SEM). Distributions were examined for normality by the Kolgorov Smirnoff, Shapiro Wilk or D'Agostino & Pearson omnibus normality test. Mann Whitney U Test was used to compare groups in instances where data were not normally distributed. Homogeneity of variances was tested graphically by examination of residual plots in SPSS and by the Brown-Forsythe test. Measures of body size were log transformed for linearity and behavioural stepwise regression data were square root transformed prior to analysis. Survival and latency to event data were analysed using Kaplan Meier survival analysis (Budaev 1997). Data were tested for differences between treatments using one-way and two-way ANOVA, or independent samples *t*-tests and multiple comparisons were made using Tukey and Bonferonni tests. Differences were considered significant at $p < 0.05$. Latency to behaviour was scored from video recordings 5 minutes at introduction and 5 minutes of each hour for the first 6 hours on days 1, 2, 4, 6 and 14 of fish fed either reference or TRP supplemented food. Data are derived from 9 replicates per feed type at T = 0. Gehan Breslow Wilcoxon analysis was used to identify differences in latency between feed treatments. Mantel Cox Log rank test for trend was used to identify trends within feed treatments over time. Kaplan Meier survival curves were compared

for differences using Gehan Breslow Wilcoxon. Stepwise multiple regression was used to identify significant predictors of behavioural and physiological data from the tests of Exp 2. Differences were considered statistically significant at $P = <0.05$.

2.4 Results Experiment 1 – Response to intruder

2.4.1 Behaviours

2.4.1.1 Initial confinement

No differences were observed for total time spent chasing an intruder by a larger resident, fed either a reference feed (7.2 $mg.g^{-1}$ TRP) or one supplemented with TRP (19.4 $mg.g^{-1}$ TRP) at 8% $BW.day^{-1}$, over the first 5 minutes of confinement at any of the days studied. See Figure 1.1 (p 43). No differences were observed for the same time period for either attack occurrence or total aggressive acts. See Figure 2.2 (p 43). No differences were observed for latency (s) to chasing or eyeballing by resident juvenile barramundi fed either a reference or TRP supplemented feed during the first 5 minutes of confinement with a 50% smaller conspecific over 1, 2, 4, 6 and 14 days. See Table 2.2 (p 44). No differences were observed for either measure on any day and no trends were present over time.

2.4.1.2 Up to 6 hours post-introduction

Mean aggressive acts and mean occurrence of attacks per 5 minutes by a larger resident barramundi toward a 50% smaller conspecific were examined over the first 6 hours of confinement. There were no differences between feed treatments for mean aggressive acts per

5 minutes on any of the days studied. See

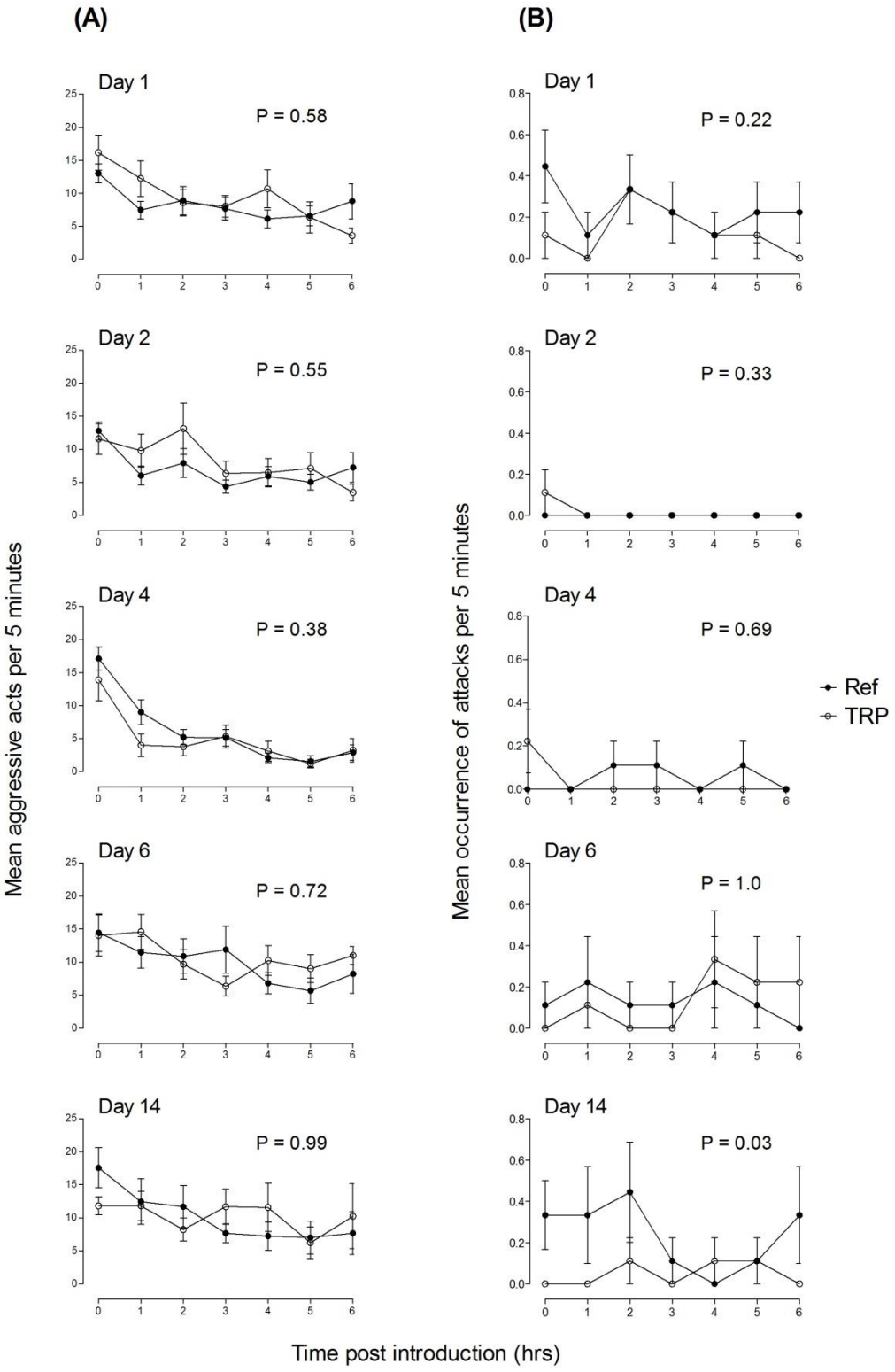

Figure 2.3 (p 45). On day 14 more attacks were perpetrated on the intruder by resident fish fed the reference feed. (F(1, 96) = 5.818, p = 0.03:

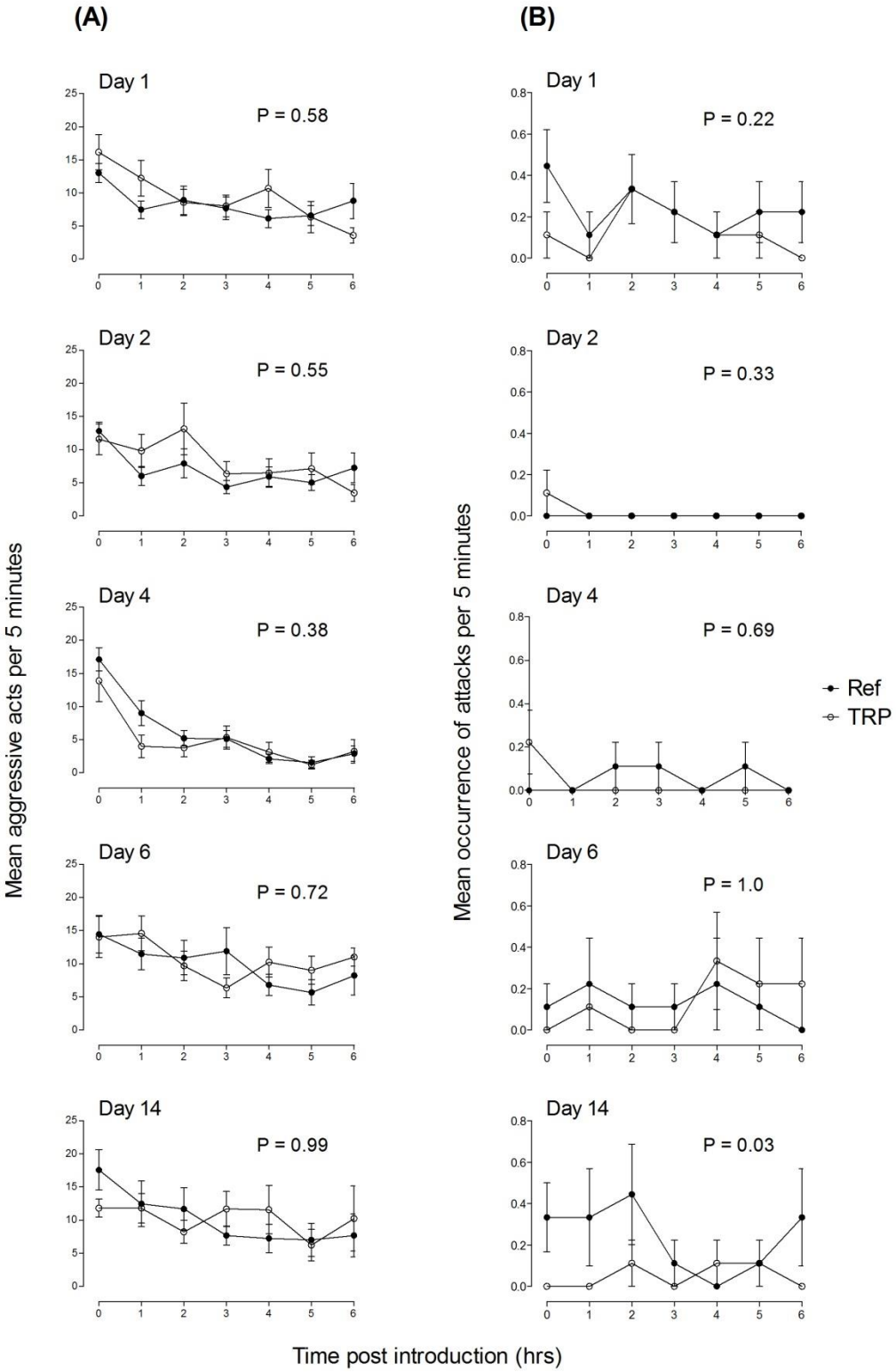

Figure 2.3, p 45).

Repeated measures ANOVA showed there were no differences ($p < 0.05$) in average duration of chase events or mean time spent chasing between fish offered different feeds. See Figure 2.4 (p 48).

Survival of the intruder, and latency to chasing and eyeballing by a larger resident barramundi toward a 50% smaller conspecific were examined over the first 6 hours of confinement. A greater latency to chasing by resident fish was observed on day 6 over the initial 6 hours of confinement for fish fed the reference feed compared to those fed the TRP supplemented feed ($x^2 = 4.52$, *df* 1, P = 0.03, Table 2.3, p 49). A greater latency to eyeballing by resident fish was observed on day 6 over the initial 6 hours of confinement for fish fed TRP supplemented feed compared to those fed the reference feed ($x^2 = 6.3$, *df* 1, P = 0.01, Table 2.3, p 49). No trends over time were observed for either feed for latency to chasing or latency to eyeballing. When survival was analysed over days 1 through 8 and day 14 no differences in survival over the first 6 hours of confinement were observed and nor were survival trends identified. See Table 2.3, (p 49) & Figure 2.5, (p 50).

2.4.1.3 Up to 24 hours post-introduction

Paired t-test, two tailed, showed no differences ($p < 0.05$) in mean time spent chasing by fish between feeds. See Figure 2.6 (p 51). Paired t-test, two tailed, showed differences ($p < 0.05$) in the mean occurrence of attacks by fish between feeds. On day 14 resident fish fed the reference feed perpetrated more attacks on intruder fish than those fed the TRP supplemented feed (Ref $\bar{x}$ = 0.65, SD = 0.07; TRP $\bar{x}$ = 0.62, SD = 0.02; t (24) = 2.24, P = 0.03; Figure 2.7, p 52).

A greater latency to eyeballing by resident fish was observed on day 1 over 24 hours of confinement for fish fed TRP supplemented feed compared to those fed the reference feed ($x^2 = 4.20$, *df* 1, P = 0.04, Table 2.4, p 53). When survival was analysed over days 1 through 8 and day 14 no differences in survival over 24 hours of confinement were observed and nor were survival trends within feed type but over time identified. See Table 2.4 (p 53) & Figure 2.8, (p 54).

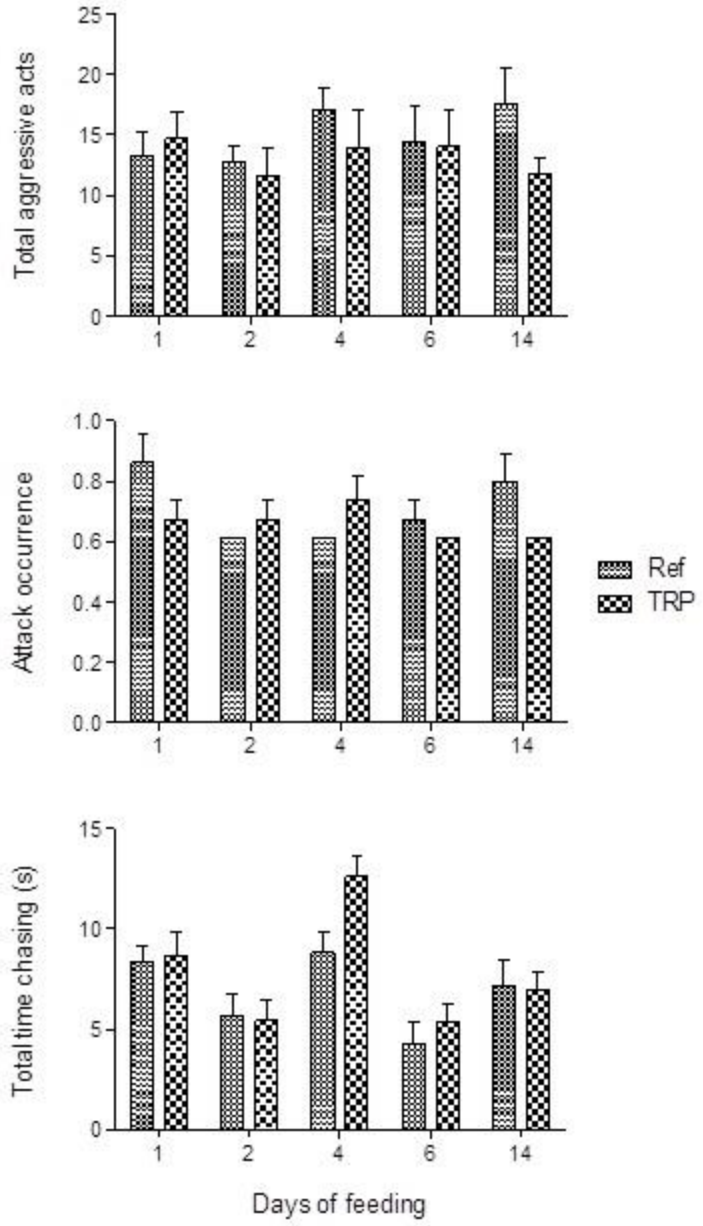

Figure 2.2 The mean ± SEM occurrence of total aggressive acts and attacks, and the mean ± SEM total time spent chasing a 50% smaller intruder barramundi over the first 5 minutes of confinement with a larger conspecific. Data were square root transformed (y=SQRT (y + 0.375)for attack occurrence and total time spent chasing. T-test showed no differences between feed treatments for any of the observed behavioural frequencies at any of the days studied.

Table 2.2 Median latency (s) to chasing and eyeballing by resident juvenile barramundi fed either a reference or TRP supplemented feed during the first 5 minutes of confinement with a 50% smaller conspecific over 1, 2, 4, 6 and 14 days. Gehan Breslow Wilcoxon analysis was used to identify differences in latency between feed treatments. Mantel Cox Log rank test for trend was used to identify trends within feed treatments over time. No differences were observed for either measure on any day and no trends were present over time as indicated by p values greater than 0.05.

		Median (s)		Chi sq	df	P
Chase latency		Ref	TRP			
Day 1		23.09	21.51	0.57	1	0.45
Day 2		43.54	81.89	1.98	1	0.16
Day 4		34.44	27.87	0.13	1	0.71
Day 6		66.12	55.81	0.14	1	0.71
Day 14		23.16	20.53	0.76	1	0.38
Trend	Ref	-	-	0.46	4	0.50
	TRP	-	-	0.09	4	0.77
Eyeball latency						
Day 1		12.27	13.01	0.08	1	0.77
Day 2		2.88	11.7	1.31	1	0.25
Day 4		22.32	36.56	2.17	1	0.14
Day 6		13.49	44.66	1.12	1	0.29
Day 14		21.48	12.74	0.02	1	0.89
Trend	Ref	-	-	0.13	4	0.71
	TRP	-	-	0.15	4	0.70

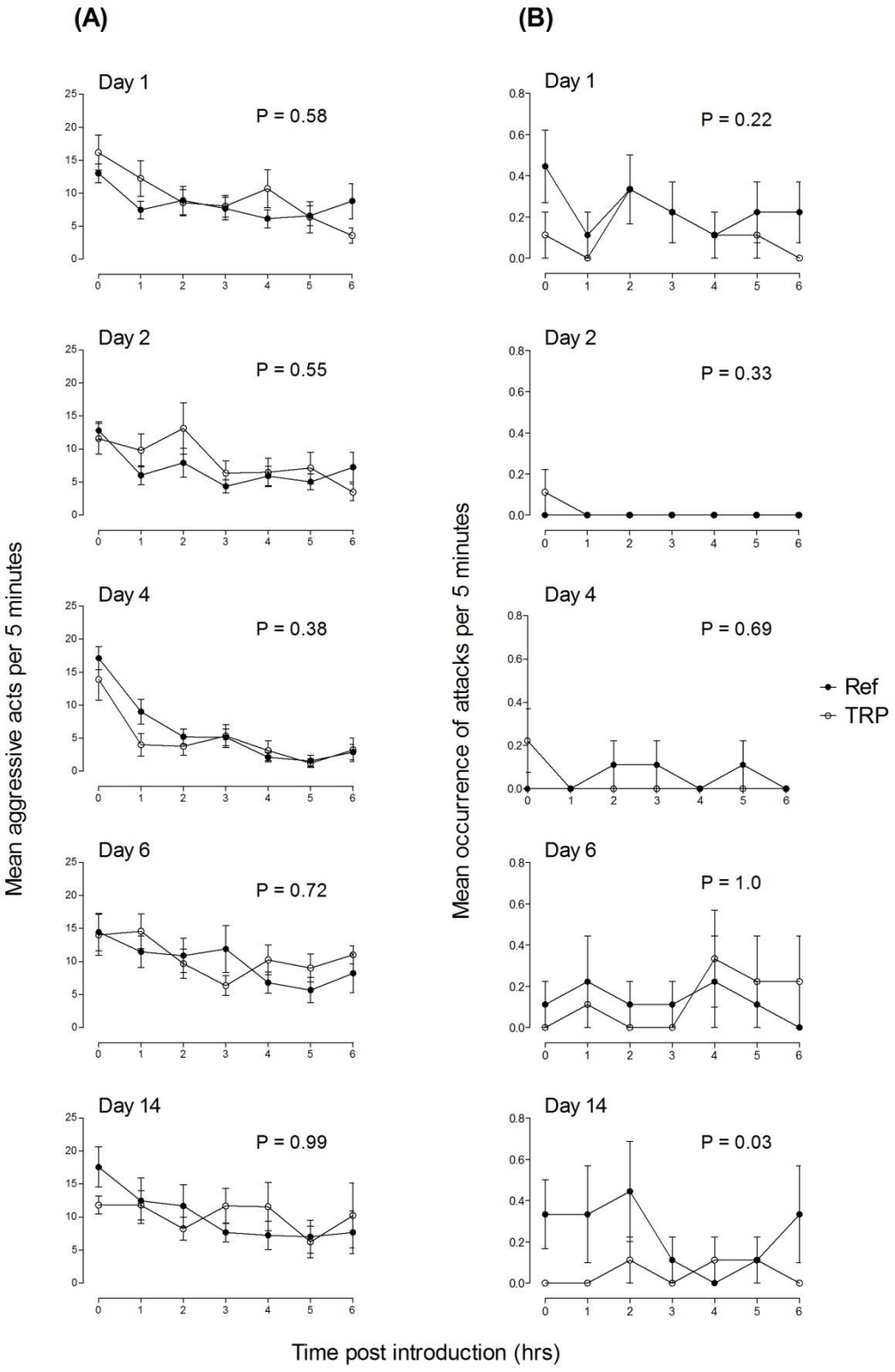
(A)
(B)
Day 1
P = 0.58
Day 2
P = 0.55
Day 4
P = 0.38
Day 6
P = 0.72
Day 14
P = 0.99
Day 1
P = 0.22
Day 2
P = 0.33
Day 4
P = 0.69
Day 6
P = 1.0
Day 14
P = 0.03
Ref
TRP
Mean aggressive acts per 5 minutes
Mean occurrence of attacks per 5 minutes
Time post introduction (hrs)

Figure 2.3 (A) Mean (± SEM, n=9) aggressive acts and (B) mean (± SEM, n=9) occurrence of attacks per 5 minutes by a larger resident barramundi toward a 50% smaller conspecific. Behaviours were scored from video recording 5 minutes at introduction and 5 minutes of each hour for the first 6 hours on days 1, 2, 4, 6 and 14 of fish fed either reference or TRP supplemented food. Data are derived from 9 replicates per feed type. Repeated measures ANOVA, showed differences ($p < 0.05$) in mean occurrence of attacks between feeds as displayed by p values.

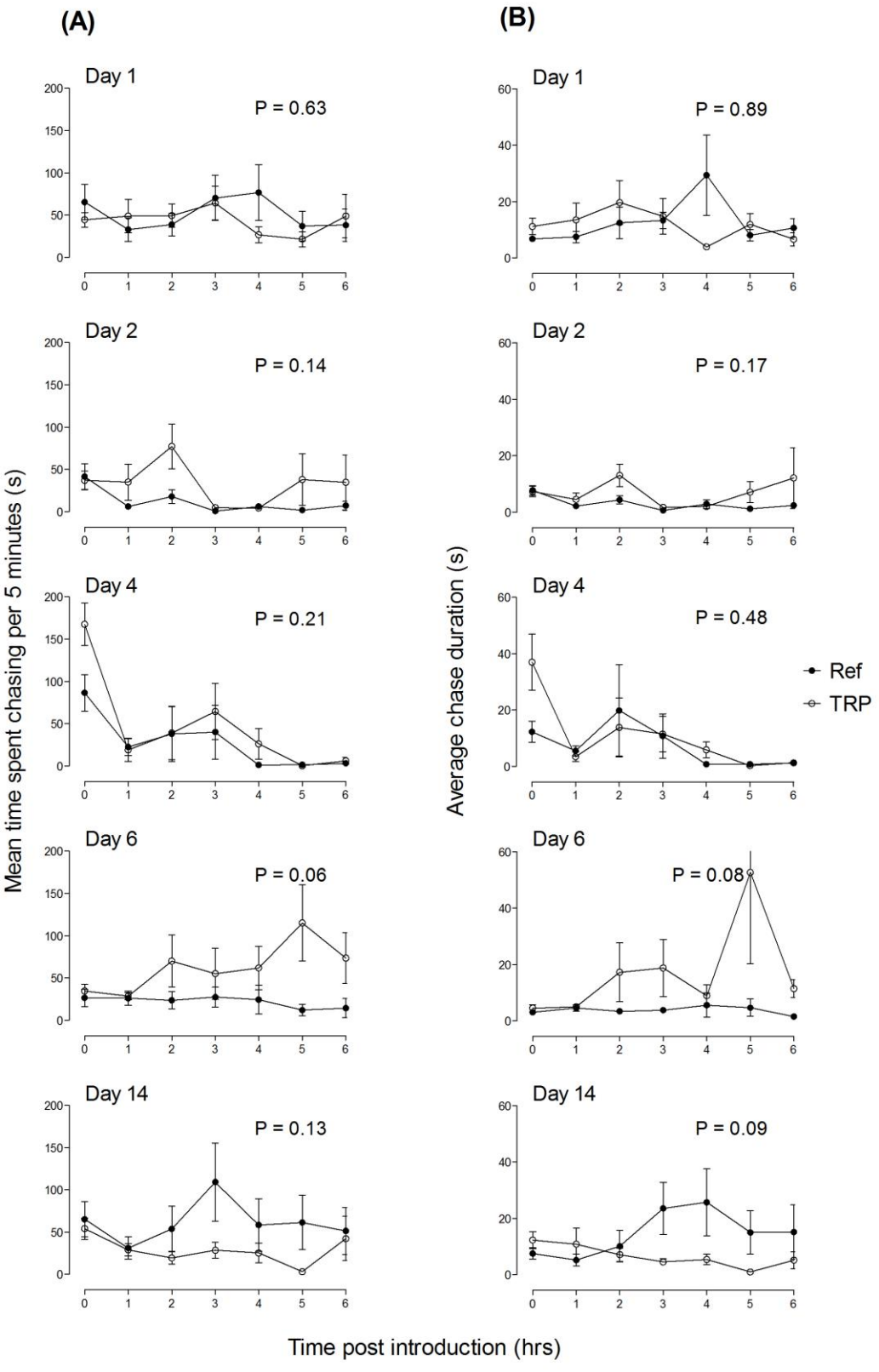
(A)
(B)
Day 1
P = 0.63
Day 1
P = 0.89
Day 2
P = 0.14
Day 2
P = 0.17
Day 4
P = 0.21
Day 4
P = 0.48
Day 6
P = 0.06
Day 6
P = 0.08
Day 14
P = 0.13
Day 14
P = 0.09
Ref
TRP
Mean time spent chasing per 5 minutes (s)
Average chase duration (s)
Time post introduction (hrs)

Figure 2.4 (A) Mean (± SEM, n=9) time spent chasing and (B) mean (± SEM, n=9) average chase duration per 5 minutes of a larger resident barramundi toward a 50% smaller conspecific. Behaviour were scored from video recording 5 minutes at introduction and 5 minutes of each hour for the first 6 hours on days 1, 2, 4, 6 and 14 of fish fed either reference or TRP supplemented food. Data are derived from 9 replicates per feed type. Repeated measures ANOVA showed no differences ($p < 0.05$) in average duration of chase events or mean time sent chasing between feeds as displayed by p values.

Table 2.3 Survival of intruder fish, and latency (s) to chasing and eyeballing by resident juvenile barramundi fed either a reference or TRP supplemented feed during the first 5 minutes of each hour over the first 6 hours of confinement with a 50% smaller conspecific over 1, 2, 4, 6 and 14 days. P value indicates days at which differences were present over the first 6 hours of confinement. Gehan Breslow Wilcoxon analysis was used to identify differences in latency between feed treatments. Mantel Cox Log rank test for trend was used to identify trends within feed treatments over time. Kaplan Meier survival curves were compared for differences using Gehan Breslow Wilcoxon. Differences were considered at P = <0.05.

		Median (s)		Chi sq	df	P
Chase latency		Ref	TRP			
Day 1		49.34	52.17	1.40	1	0.24
Day 2		191.1	85.73	3.24	1	0.07
Day 4		100.6	39.32	2.33	1	0.13
Day 6		81.19	32.69	4.52	1	0.03*
Day 14		42.38	54.25	0.78	1	0.38
Trend	Ref	-	-	0.92	4	0.34
	TRP	-	-	2.45	4	0.12
Eyeball latency						
Day 1		12.27	33.6	3.83	1	0.05
Day 2		32.31	22.05	0.50	1	0.48
Day 4		50.58	41.78	1.94	1	0.16
Day 6		11.28	39.53	6.30	1	0.01*
Day 14		19.09	23.97	0.75	1	0.39
Trend	Ref	-	-	0.67	4	0.41
	TRP	-	-	0.00	4	0.99
Survival						
Day 1		-	-	0.15	1	0.70
Day 2		-	-	1.07	1	0.30
Day 3		-	-	2.12	1	0.15
Day 4		-	-	3.00	1	0.08
Day 5		-	-	1.00	1	0.32
Day 6		-	-	3.38	1	0.07
Day 7		-	-	3.38	1	0.07
Day 8		-	-	0.46	1	0.50
Day 14		-	-	0.14	1	0.71
Trend	Ref	-	-	2.37	8	0.12
	TRP	-	-	2.31	8	0.13

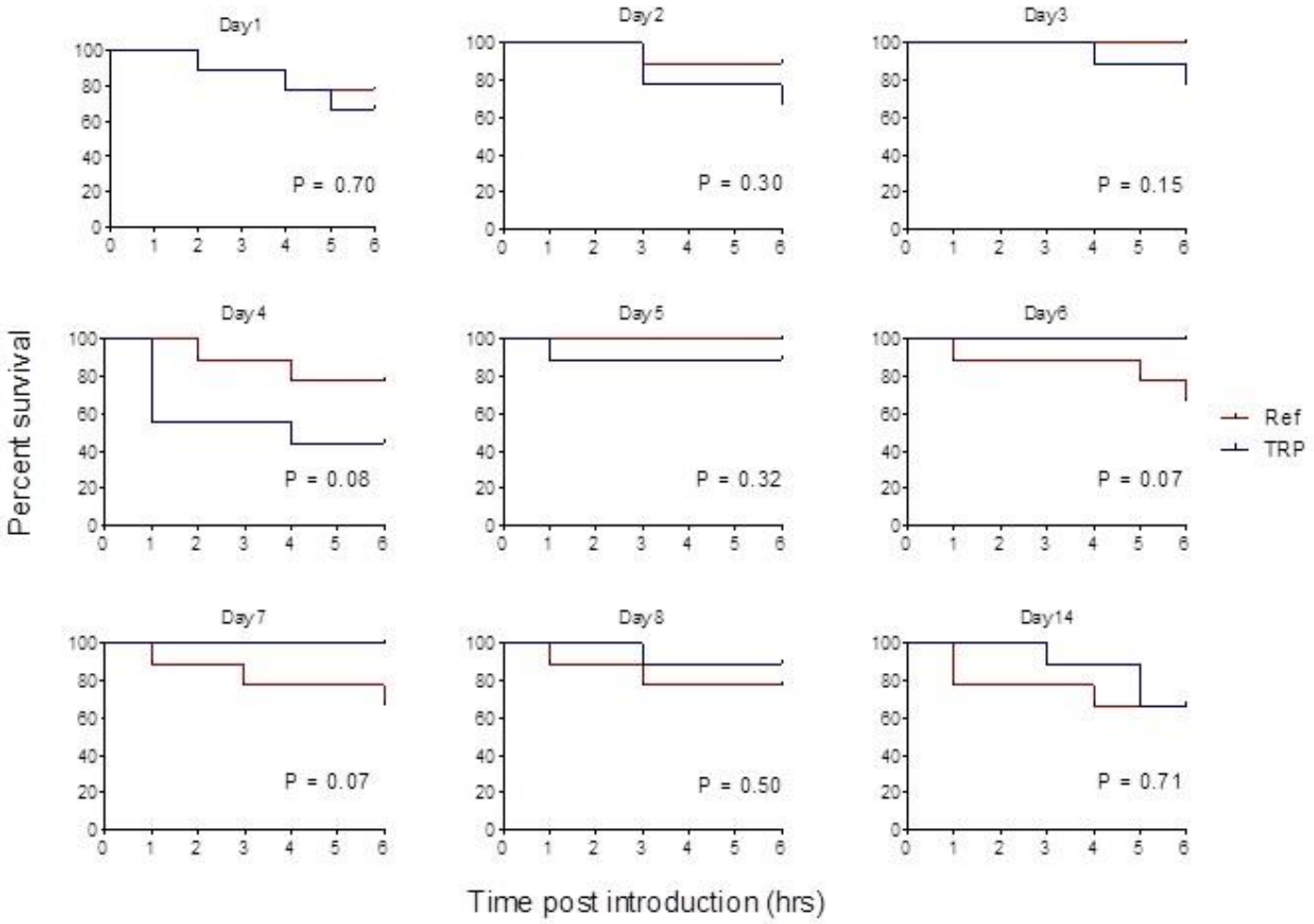

Figure 2.5 Percent survival over the first 6 hours of 50% smaller intruder barramundi exposed to larger resident barramundi for 24 hours. N = 9 per feed type at T = 0 and fish were delivered reference or TRP supplemented feed over 1 through 8 and 14 days. No differences in survival were observed between feed type (Ref or TRP) over the initial 6 hours of confinement.

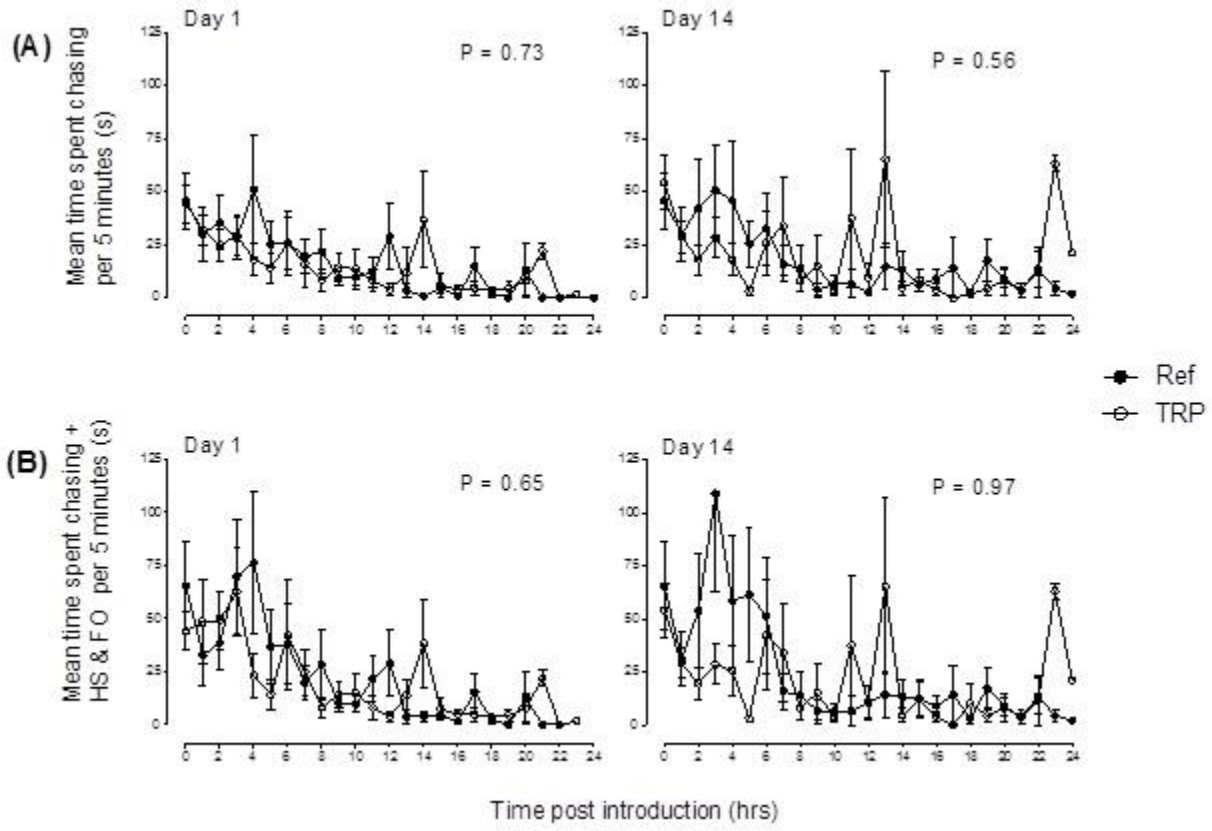

Figure 2.6 (A) Mean (± SEM, n=9) time (s) spent chasing (without other behaviours) per 5 minutes, and (B) mean (± SEM, n=9) time spent chasing including whilst headshaking (HS) and with fast opercula (FO), a 50% smaller intruder conspecific. Behaviours were scored from video recordings of 5 minutes at introduction and 5 minutes of each hour for 24 hours, after both 1 and 14 days of either reference or TRP supplemented food. Data are derived from 9 replicates per feed type. Distributions were square root transformed (y=SQRT (y + 0.375)for normality but presented untransformed. Paired t-test, two tailed, showed no differences ($p < 0.05$) between feeds in average time spent chasing as displayed by p values.

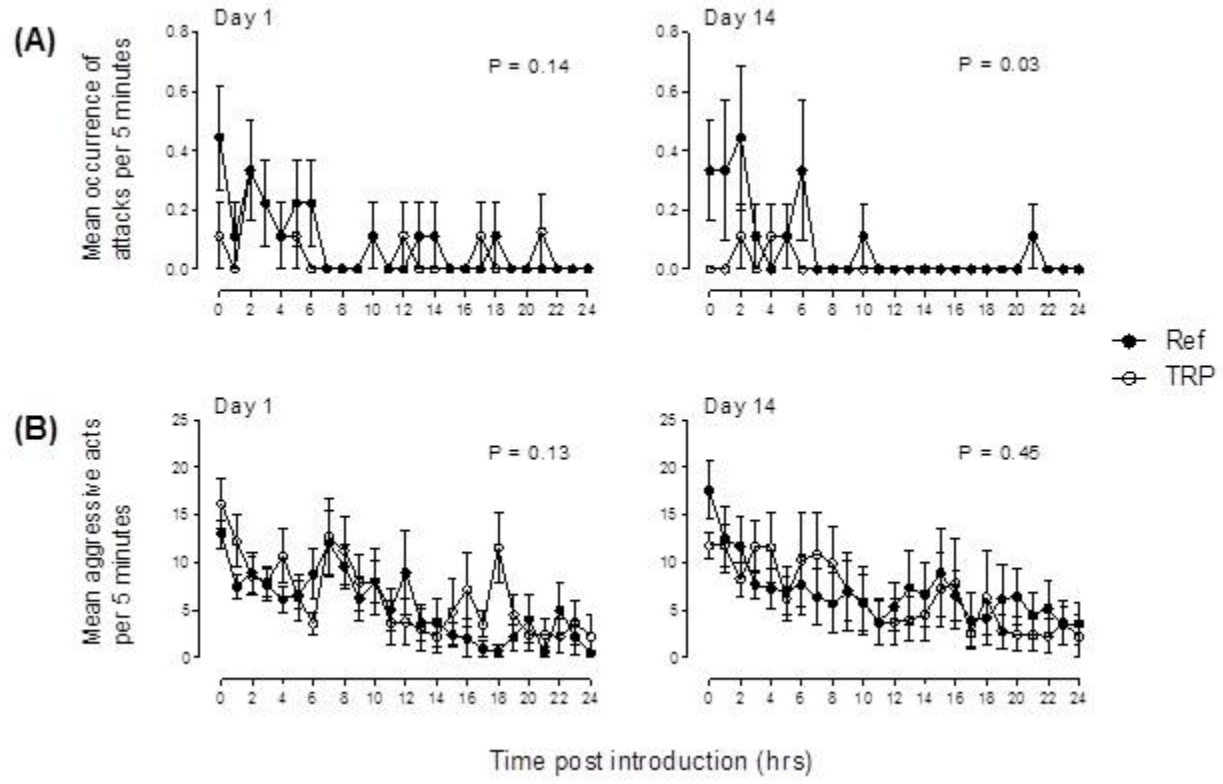

Figure 2.7 (A) Mean (± SEM, n=9) aggressive acts and (B) mean (± SEM, n=9) occurrence of attacks per 5 minutes by a larger resident barramundi toward a 50% smaller conspecific. Behaviours were scored from video recordings of 5 minutes at introduction and 5 minutes of each hour for 24 hours, after both 1 and 14 days of either reference or TRP supplemented food. Data are derived from 9 replicates per feed type. Distributions were square root transformed (y=SQRT (y + 0.375)for normality but presented untransformed. Paired t-test, two tailed, showed a difference ($p < 0.05$) between feeds in the mean occurrence of attacks on day 14 as displayed by p value.

Table 2.4 Survival of intruder fish, and latency (s) to chasing and eyeballing, by resident juvenile barramundi fed either a reference or TRP supplemented feed during 5 minutes at introduction, and the first 5 minutes of each hour over 24 hours of confinement with a 50% smaller conspecific over 1, 2, 4, 6 and 14 days. P value indicates days at which differences were present over the first 6 hours of confinement. Gehan Breslow Wilcoxon analysis was used to identify differences in latency between feed treatments. Mantel Cox Log rank test for trend was used to identify trends within feed treatments over time. Kaplan Meier survival curves were compared for differences using Gehan Breslow Wilcoxon. Differences were considered at P = <0.05.

		Median (s)		Chi sq	df	P
Chase latency		Ref	TRP			
Day 1		69.6	57.62	0.34	1	0.56
Day 14		68.36	66.65	0.00	1	0.95
Eyeball latency						
Day 1		13.81	29.15	4.20	1	0.04*
Day 14		23.42	28.58	2.15	1	0.14
Survival						
Day 1		-	-	0.00	1	0.96
Day 2		-	-	1.40	1	0.24
Day 3		-	-	2.79	1	0.09
Day 4		-	-	1.43	1	0.23
Day 5		-	-	0.74	1	0.39
Day 6		-	-	0.26	1	0.61
Day 7		-	-	0.04	1	0.85
Day 8		-	-	0.14	1	0.71
Day 14		-	-	0.02	1	0.89
Trend	Ref	-	-	1.78	1	0.18
	TRP	-	-	0.01	1	0.93

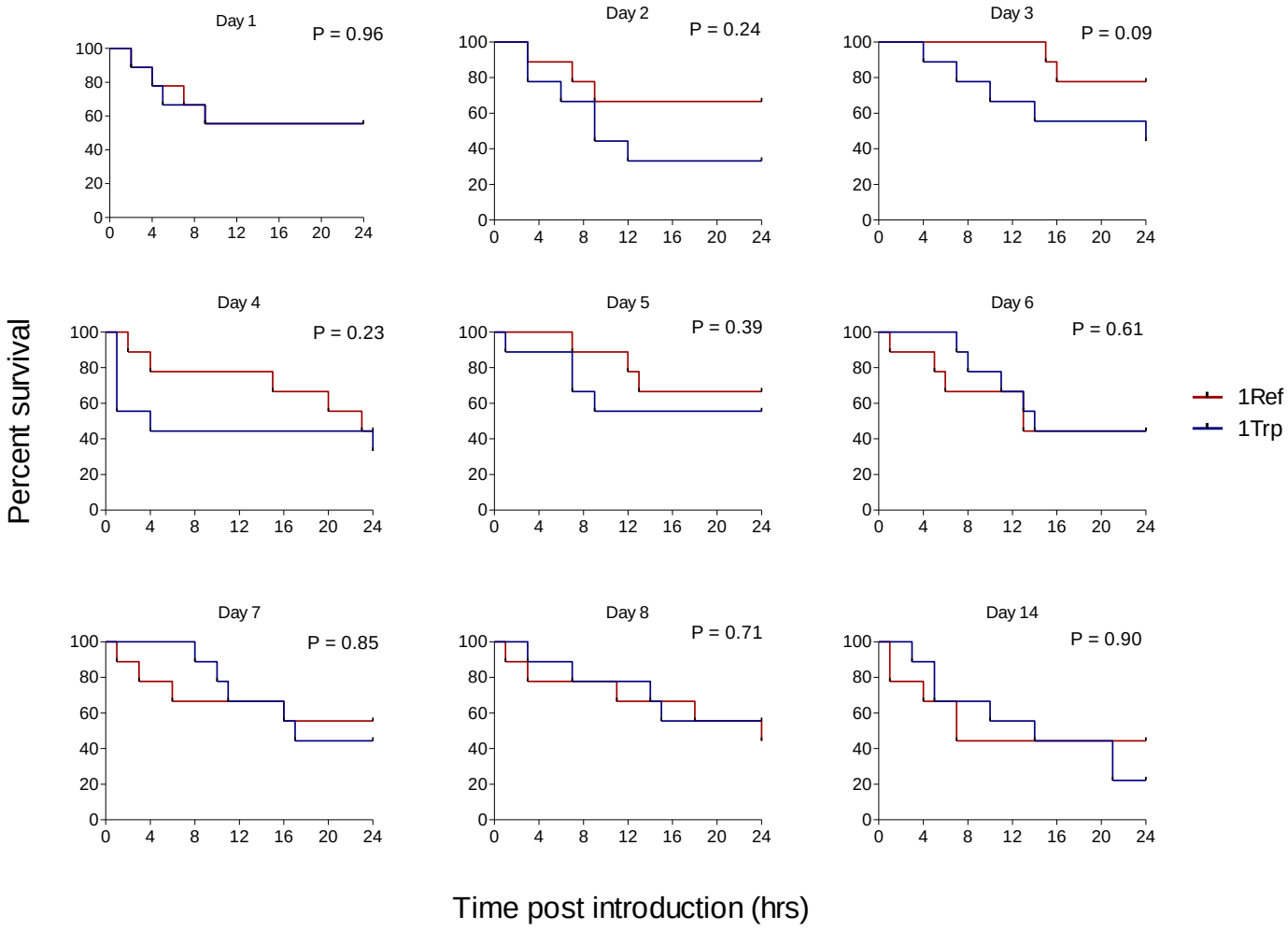

Figure 2.8 Percent survival of 50% smaller intruder barramundi exposed to larger resident barramundi for 24 hours. N = 9 per feed type at T = 0 and fish were delivered reference or TRP supplemented feed over 1 through 8 and 14 days. No differences in survival were observed between feed type (Ref or TRP) over 24 hours of confinement.

2.5 Results Experiment 2 – Behavioural type

A series of forwards stepwise multiple regressions were performed to predict the influence of variables upon one another both across (Table 2.5, p 58) and within (Table 2.6, p 59), the three behavioural tests (mirror, foreign object, intruder), orientation of the resident, and physiological stress response data. The resulting models are used to identify physiological response associated with behavioural type, and / or consistent responses to variable stimuli. Machine error caused brain serotonergic data to be unusable and thus these data were not included and are no longer referred to.

2.5.1 Across test multiple regressions

A significant regression equation was found for chasing the intruder ($F = 9.48$, *df* 4, 35; $P < 0.0001$), with an R^2 of 0.52, showing that the 4 predictors account for 52% of the variance in total time spent chasing the intruder. The final model states that time spent chasing the intruder is equal to -124.010 + 0.254 (Facing FO) + 0.997 (Eyeball FO) + 0.252 (Facing left wall) + 0.002 (Facing mirror). All independent variables in this model were significant predictors of time spent chasing an intruder. The strongest predictor is total time spent eyeballing the FO: a 1 point increase in time spent eyeballing the FO is associated with a 0.997 point increase in time spent chasing an intruder.

A significant regression equation was found for ignoring the intruder ($F = 7.002$, *df* 1, 38; $P < 0.05$), with an R^2 of 0.156, showing that the predictor accounts for 15.6 % of the variance in total time spent ignoring the intruder. The final model states that time spent ignoring the intruder is equal to -556.12+ 7.411 (facing right wall).

A significant regression equation was found for the occurrence of attacking the mirror ($F = 9.275$, *df* 3, 35; $P < 0.0001$), with an R^2 of 0.443, showing that the 3 predictors account for 44.3 % of the variance in the number of attacks on a mirror. The final model states that the number

of attacks on a mirror is equal to -4.709 + 5.786 (Blood lactate) – 2.31 x 10^{-5} (Eyeballing intruder)– 4.16 x 10^{-6} (Ignoring intruder) All independent variables in this model were significant predictors of the number of attacks on a mirror. The strongest predictor is blood lactate concentration: a 1 point increase in blood lactate is associated with a 5.786 point increase in the occurrence of attacks on a mirror.

A significant regression equation was found for the occurrence of eyeballing a FO ($F = 5.654$, *df* 1, 37; $P < 0.05$), with an R^2 of 0.133, showing that the predictor accounts for 13.3 % of the variance in the occurrence of eyeballing a FO. The final model states that time spent ignoring the intruder is equal to 12.439 + 0.322 (Eyeballing intruder).

A significant regression equation was found for the time spent eyeballing a FO ($F = 6.287$, *df* 2, 36; $P < 0.01$), with an R^2 of 0.259, showing that the 2 predictors account for 25.9 % of the variance in the time spent eyeballing a FO. The final model states that the time spent eyeballing a FO is equal to 52.23 + 0.234 (Chasing intruder) – 0.107 (Facing left wall). Both independent variables in this model were significant predictors of the time spent eyeballing a FO. The strongest predictor is chasing an intruder: a 1 point increase in time spent chasing an intruder is associated with a 0.234 point increase in the time spent eyeballing a FO.

A significant regression equation was found for blood lactate ($F = 4.276$, *df* 1, 38; $P < 0.05$), with an R^2 of 0.101, showing that the predictor accounts for 10.1 % of the variance in blood lactate concentration of the intruder. The final model states that time spent ignoring the intruder is equal to 1.745 + 2.34 x 10^{-6} (Facing left wall).

2.5.2 Within test multiple regressions

A significant regression equation was found for the occurrence of failed attacks ($F = 21.632$, *df* 5, 34; $P < 0.0001$), with an R^2 of 0.761, showing that the 5 predictors account for 76.1 % of the variance in the occurrence of failed attacks. The final model states that the occurrence of failed attacks is equal to -0.241 + 0.164 (Eyeball intruder N) – 1.11 x 10^{-5} (Eyeball intruder TT) – 6.84 x

10^{-6} (Chasing intruder) – 2.31 x 10^{-6} (Observe intruder) – 2.66 x 10^{-5}(Wall swimming) All independent variables in this model were significant predictors of the occurrence of failed attacks. The strongest predictor is the occurrence of eyeballing the intruder: a 1 point increase in the occurrence of eyeballing is associated with a 0.164 point increase the occurrence of failed attacks.

A significant regression equation was found for blood glucose (F = 21.91, *df* 1, 38; P < 0.0001), with an R^2 of 0.366, showing that the predictor accounts for 33.6 % of the variance in the concentration of blood glucose. The final model states that blood glucose concentration is equal to 3.556 + 3.28 x 10^{-5}(Chased by intruder).

A significant regression equation was found for time spent being eyeballed by the intruder (F = 929.76, *df* 1, 38; P < 0.0001), with an R^2 of 0.961, showing that the predictor accounts for 96.1 % of the variance in the time spent being eyeballed by the intruder. The final model states that time spent being eyeballed by the intruder is equal to 225.629 + 0.725 (Chased by intruder).

Table 2.5 Dependent behavioural and physiological variables and their significant predictors as defined by across test multiple stepwise regressions. The R^2 value describes the proportion of variance attributed to the model. Durbin Watson statistic of close to 2 describes the independence of all the variables from one another. All models are significant ($p < 0.05$)

Dependent variable	Predictors	F	df	Sig	R2	B	Beta	Durbin Watson
Chasing intruder [I] (TT)	Facing FO [FO] (TT)	9.48	4, 35	0	0.52	0.254	0.370	2.227
	Eyeball FO [FO] (TT)					0.997	0.462	
	Facing left wall [M] (TT)					0.252	0.431	
	Facing mirror [M] (TT)					0.002	0.302	
Ignoring intruder [I] (TT)	Facing right wall [FO] (TT)	7.002	1, 38	0.012	0.156	7.411	0.394	2.122
Blood lactate [I]	Facing left wall [M] (TT)	4.276	1, 38	0.046	0.101	2.34E-06	0.318	2.212
Attack mirror [M] (N)	Blood lactate [I]	9.275	3, 35	0	0.443	5.786	0.643	1.644
	Eyeballing intruder [I] (TT)					-2.31E-05	-0.322	
	Ignoring intruder [I] (TT)					-4.16E-06	-0.321	
Eyeballing FO [FO] (N)	Eyeballing intruder [I] (N)	5.654	1, 37	0.023	0.133	0.322	0.364	2.127
Eyeballing FO [FO] (TT)	Chasing intruder [I] (TT)	6.287	2, 36	0.005	0.259	0.234	0.500	2.076
	Facing left wall [M] (TT)					-0.107	-0.396	

Table 2.6 Dependent behavioural and physiological variables and their significant predictors as defined by within test multiple stepwise regressions. The R^2 value describes the proportion of variance attributed to the model. Durbin Watson statistic of close to 2 describes the independence of all the variables from one another. All models are significant ($p < 0.05$)

Dependent variable	Predictors	F	df	Sig	R2	B	Beta	Durbin Watson
Failed attack [I] (N)	Eyeball [I] (N)	21.632	5, 34	0	0.761	0.164	1.528	2.153
	Eyeball [I] (TT)					-1.11E-05	-1.027	
	Chasing intruder [I] (TT)					-6.84E-06	-0.401	
	Observe intruder [I] (TT)					-2.31E-06	-0.326	
	Wall swimming [I] (TT)					-2.66E-05	-0.241	
Blood Glucose [I]	Chased by intruder [I] (TT)	21.91	1, 38	0	0.366	3.28E-05	0.605	1.574
Eyeballed by intruder [I] (TT)	Chased by intruder [I] (TT)	929.76	1, 38	0	0.961	0.725	0.98	2.04

2.6 Discussion

This study is the first to quantify aggressive behaviour in barramundi. Though a number of studies have examined intracohort cannibalism by barramundi, often in relation to morphology and mitigation (Parazo *et al.* 1991; Qin *et al.* 2004; Appelbaum and Arockiaraj 2010; Arockiaraj and Appelbaum 2011; Ribeiro and Qin 2013; Ribeiro and Qin 2016), none have examined aggressive behaviours associated with cannibalism such as eyeballing, chasing and attacking. This study is the first to examine the effects of supplementary dietary TRP on intracohort interactions between juvenile barramundi. Whilst small isolated effects were observed, overall the supplementation of feed with TRP to a level of 19.4 $mg.g^{-1}$ had no perceived effect on aggressive behaviours or incidence of cannibalism in juvenile barramundi pairs.

Data were presented across the total 24 h period of dyadic observation, as well as the first 6 hours and the initial 5 minutes of confinement. The 6 h observations provided behavioural data thought to be less likely influenced by hunger, as feeding was conducted immediately prior to intrusion, and also a higher and more consistent replication as survival was effected by time. The 5 minute observation provided an important snapshot of the initial response to an intruder. Whilst short-, medium-, and long-term observations provide an interesting window on aggressive interactions and their effects between juvenile barramundi, the 6 hour observations were considered to be more useful from an application perspective with particular reference to feeding frequency. Given the described agonistic interactions amongst barramundi are partially attributed to hunger, it is common practice to feed fish of this size 3 times per day. Therefore, under 12 : 12 L : D photoperiod there would be a maximum of 6 hours between feeds during the light phase.

Latency to event data has been presented by Kaplan Meier survival analysis (Budaev 1997). Measures of latency to state (behaviours that occur over time) data can sometimes provide a misleading picture, especially when calculated as a mean from brief windows over a longer duration as in the current study. Firstly the initial time point reflects an initial response to the intruder by the resident, however latency at later time points is more reflective of the rate of occurrence of the event as the beginning of the time window is arbitrary. It is probably more

reflective of gross aggressive response when measured from the point of intrusion. Secondly, the rate of occurrence and thus latency to event may change over time, possibly as a result of increasing familiarity or hunger. A greater latency until chasing commences in either circumstance may be interpreted that less chasing occurred however there may have been fewer individual chases but with a longer duration. Therefore duration of state behaviours should always be considered when discussing their latencies. No differences were observed when latency to either chasing or eyeballing was examined over the same time period.

No differences were recorded between feed types for observed behaviours over the first 5 minutes of confinement. Attack occurrence, the number of total aggressive acts and the total time spent chasing did not differ between feed types at any of the days examined. Indeed, these data were not only uniform between feed type but also across days. When latency to event (chasing and eyeballing) data were examined no differences between feed type were observed on any day for either parameter. Furthermore when latency curves were compared for trends over time none were observed. Survival in both groups over the first five minutes of confinement was 100% on all days. Together these results show that supplementary dietary TRP added at 14 $mg.g^{-1}$ and fed over 1, 2, 4, 6 and 14 days had no effect on attack occurrence, total time spent chasing, the total number of aggressive acts, latency to chasing, latency to eyeballing, or survival over the initial 5 minutes when a larger resident barramundi was confined with a 50% smaller conspecific.

When data were examined over the first 6 hours of confinement after 6 days of food consumption, TRP supplemented feed was found to increase latency to first eyeballing event, however latency to chasing was reduced and time spent chasing was increased over fish fed the reference feed. Survival was not affected. On day 14, during the first 6 hours, there were more attacks on the intruder by fish fed the reference feed. Once more survival was not affected.

When data were examined over the full 24 hours of confinement there was a higher latency to eyeballing for fish fed the TRP supplemented feed on day 1 and a greater occurrence of attacks by fish fed the reference feed on day 14.

These results could be viewed as a trend toward a reduction in agonistic behaviour after 6 days of receiving a TRP supplemented feed, which would be broadly comparable to research on rainbow trout (Winberg *et al.* 2001). Other studies present benefits of TRP supplementation at greater than 10 days of ingestion, however no data is presented for earlier time points (Hseu *et al.* 2003; Leopoldo *et al.* 2010). There was a trend toward a greater time spent chasing by fish fed a TRP supplemented feed over days 1 to 6 inclusive. The mean time TRP supplemented feed residents spent chasing intruders was 13.6, 32.9, 46.0 and 62.7 seconds for days 1, 2, 4 and 6 respectively. Corresponding data for fish fed the reference feed was 17.1, 11.5, 27.5 and 22.1 seconds. These data are extracted from Figure 2.4, (p 48). On day 14 the trend was reversed with fish fed the reference feed recording longer mean chase duration, and perpetrating more attacks on intruders, than those fed the TRP supplemented feed. These apparently conflicting results might suggest an initial antagonistic effect of TRP supplementation of feed followed by a suppression of motivation to chase a smaller conspecific. This suppression of motivation may be via an effect on the desire for locomotor activity, such as lethargy, drowsiness or nausea, themselves factors that are often associated with reduced food intake, a phenomenon observed in later experiments. Active behaviour in rats was found to reduce after intraperitoneal (IP) injections of 20 $mg.Kg^{-1}$ TRP, and also that concurrent intra-peritoneal (IP) administration of methionine attenuated these results indicating the role of other amino acids on behavioural effects of TRP (Taylor 1976).

It is tempting to conclude that this provides evidence of the neurologically calmative effect of elevated brain [5-HT] and concurs with a delayed behavioural response attributed to elevated dietary TRP (Winberg *et al.* 2001; Hseu *et al.* 2003; Leopoldo *et al.* 2010). This conclusion does not address the trend for an increase in time spent chasing, culminating in a longer total duration of chasing on day 6 by fish fed the TRP supplemented feed. In the current study the

difference in occurrence of attacks between the two feed treatments on day 14 (

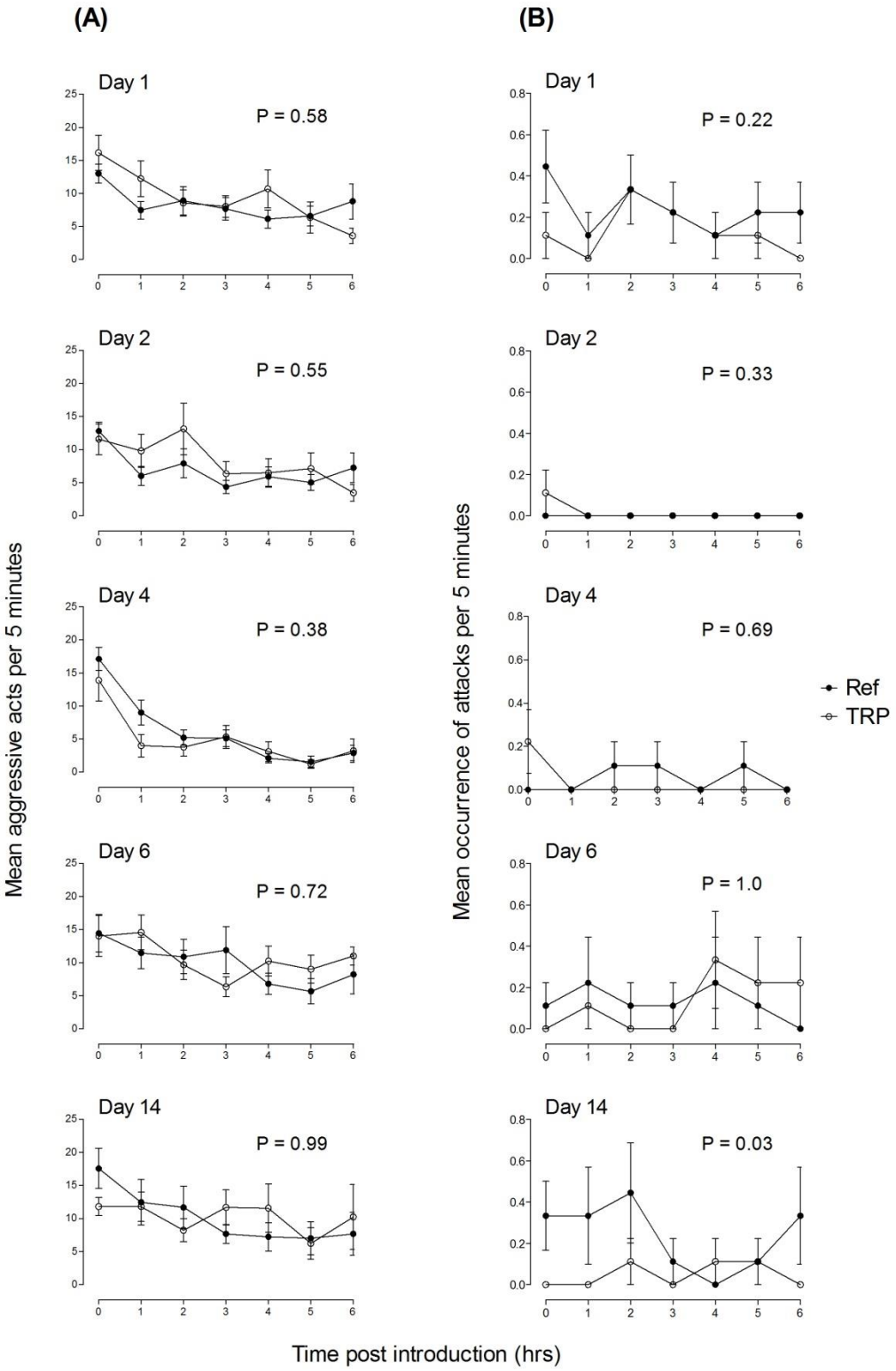

Figure 2.3, p 45) appears more reflective of an increase in attacks by fish fed the reference feed, rather than a reduction in occurrence by fish fed the TRP supplemented feed (

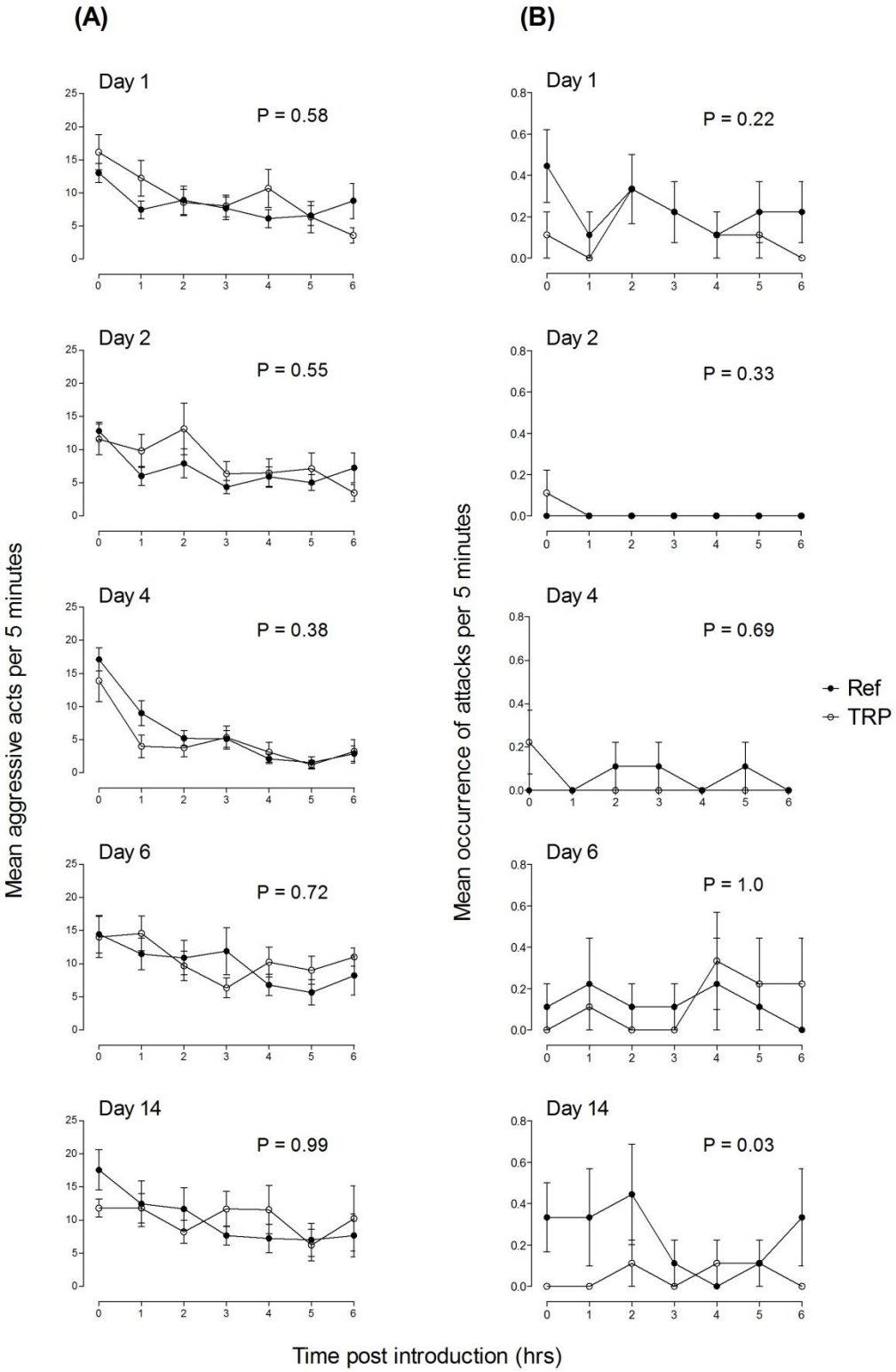

Figure 2.3, p 45. Furthermore, at P = 0.04, the effect is only just significant. It could be perceived either that a 14 day treatment of TRP supplemented feed delivered a calmative effect, preventing the elevation of attack behaviour observed on day 14 in fish fed the reference feed. Alternatively, it is also possible that by day 14 the fish had developed an increased level of hunger. In the current study fish were fed at 8% of body weight per day, as an expected satiation ration for fish of this size, however more recent studies by the author (Chapter 3, p 77) have determined a much higher satiation feed intake for barramundi of this size. The hypophagic effect of supplementary dietary TRP may have masked the effect of hunger and thus contributed to a reduction in time spent chasing the intruder.

The hypophagic effect of serotonin on fish is well documented (Johnston *et al.* 1990; Winberg *et al.* 1993; De Pedro *et al.* 1998a; Lin *et al.* 2000; Ruibal *et al.* 2002; Ortega *et al.* 2013; Pérez-Maceira *et al.* 2016) however in most cases food intake comparison was not an aim of the experiment, more an incidental report. No differences in food intake of rainbow trout were observed when feeds were supplemented with TRP at up to 5.9 $mg.g^{-1}$ (a much lower inclusion than the current experiment 1) and delivered for up to 98 days (Johnston *et al.* 1990; Lepage *et al.* 2002; Lepage *et al.* 2003). A higher supplementary inclusion of 15.0 $mg.g^{-1}$ TRP (similar to the current experiment 1) also failed to have an impact on food intake in rainbow trout over 3 or 7 days (Winberg *et al.* 2001). None of these experimental designs detail whether fish were fed to satiation. In another experiment on rainbow trout, fish were fed to satiation with feed containing 24.7 $mg.g^{-1}$ TRP over 77 days and food intake was found to increase, though growth (SGR) and efficiency (FCR) were reduced (Papoutsoglou *et al.* 2005). Coloso *et al.* (2004a) found that barramundi fed a restricted ration had reduced growth (SGR) and efficiency (FER) at very low (1.1 $mg.g^{-1}$) total TRP inclusion, presumably as TRP became limiting rather than as an effect of 5-HT induced hypophagia, but didn't test any feed with more than 6.1 $mg.g^{-1}$ TRP. Protein synthesis typically sequesters approximately half of available TRP (Kalyanasundaram and Ramanamurthy 1983). A reduction in growth was also noted in orange spotted grouper (*Epinephelus coioides*) fed to satiation with feed supplemented at 2.5, 5.0 and 10.0 $mg.g^{-1}$ TRP over 10 days however no data analysis of food consumption were provided (Hseu *et al.* 2003). Apparently a negative effect of TRP supplementation at 5, 7.5 and 10 $mg.g^{-1}$ on growth in mud

crabs (*Scylla serrata*) was evident however data appear contradictory as the presented table displays no differences with the control group (Leopoldo *et al.* 2010). An increase in food intake was observed in brown trout (*Salmo trutta*) fed TRP supplemented feed at 3.0 $mg.g^{-1}$, however food intake was quantified by a feeding response score, rather than against satiation (Hoglund *et al.* 2007). A further study on rainbow trout showed an elevation in telencephalic levels of 5OH-IAA and 5OH-IAA : 5HT following long term food deprivation, further suggesting the role of the serotonergic system in appetite mediation and intake control (Ruibal *et al.* 2002). The use of 5OH-IAA, a decay product of 5-HT, as a suitable measurement, rather than 5-HT has however been questioned (Kiser *et al.* 2012). The hypophagic effect is at least partially mediated via 5-HT receptors located in various medial hypothalamic nuclei (Leibowitz and Alexander 1998), whilst pre-treatment with the corticotropin-releasing factor (CRF) antagonist α-CRF_{9-41} partially blocked the hypophagia in goldfish, suggesting CRF may have a role in mediating a 5-HT induced reduction in food intake (De Pedro *et al.* 1998a).

CRF is part of a family of peptides which in teleost fish also includes three urotensins UTn1, UTn2 and UTn3, and is found in all vertebrates. The stress response in fish is characterized by hypothalamic CRF release which stimulates pituitary adrenocorticotropic hormone (ACTH) in the pituitary gland via neurosecretory fibres, and subsequent glucocorticoid synthesis and release from the interrenal tissue. CRF synthesis is primarily via peptidergic neurons in the nucleus preoptic (NPO) of the hypothalamus, however is also expressed in extrahypothalamic brain regions and in peripheral tissues such as the caudal neurosecretory system (Flik G *et al.* 2006). The eventual CRF signal is determined by the CRF structure, the presence of CRF binding protein (CRF-BP), and the CRF receptor (CRF-R1 or CRF_R2). All of the CRF-like peptides are thought to bind and consequently their function may be modified by CRF-BP (Ronan and Summers 2011). The effects of CRF have been shown to be mediated by receptor type with CRF-R1 reported to mediate the activation of the HPI axis (Huising *et al.* 2004) and to induce anxiety like behaviour in mice (Heinrichs *et al.* 1997). CRF-R2 have been implicated in anxiety control in mice (Bale *et al.* 2000) as well as a number of other behavioral and physiological adaptations to stress response such as prolonged hypophagia and associated weight loss (Coste *et al.* 2006). Furthermore ICV administration of peptides from the CRF group have been shown

to inhibit food intake in a dose dependent manner in goldfish (De Pedro *et al.* 1993; Bernier and Peter 2001a), and feeding suppression in hypoxic rainbow trout has also been partially attributed to increased CRF-related peptide activity (Bernier and Craig 2005). More recent research on cortisol-administered, chronically stressed rainbow trout also implicates NPO CRF as well as liver leptin, a hormone associated with satiety, as mediators of reduced food intake (Madison *et al.* 2015). Unsurprisingly, in such an evolutionarily conserved system with numerous outcomes, CRF synthesis is not only stress related. Studies on rats have shown that 5HT stimulates CRF release, which interacts with 5HT receptors on CRF neurons in the paraventricular nucleus PVN (homolog to the NPO in fish), and activates CRF synthesis both in vitro (Nakagami *et al.* 1986; Itoi *et al.* 1998) and in vivo (Kageyama *et al.* 1998). Studies on teleost fish are more limited, however Medeiros *et al.* (2014) demonstrated an increase in hypothalamic CRF precursor DNA and subsequent pituitary ACTH release in toadfish (*Opsanus beta*) after intravenous injection of the $5HT_{1A}$ receptor agonist, 8-OH-DPAT.

Numerous, predominately mammalian studies, describe behavioural modulation relative to the serotonergic system in areas such as parental attachment and caregiving (Caspi *et al.* 2003), social play (Suomi 2006), adult social perceptions (Beacher *et al.* 2011), mating behaviour (Kravitz 2000), social hierarchy establishment (Chiao 2010), cooperation (Higley *et al.* 1996), and aggression (Kiser *et al.* 2012). The term aggression covers a broad spectrum of behaviours, each motivated by diverse causes and with often disparate goals. Moyer (1976) recognized 7 categories of animal aggression with subsequent evidence for their independence supported by observation of activation of different brain regions and neurochemical characteristics. The categories of predatory, inter-male, territorial, maternal, irritable, fear induced and instrumental, as identified by Moyer, can be grouped into predatory and affective subtypes (van Erp and Miczek 1997; Perreault *et al.* 2003), where predatory, or proactive aggression is offensive, with the aim of securing a reward, whilst affective, or reactive aggression is defensive, with the aim of avoiding a negative outcome. The importance of defining aggressive acts should not be overlooked as differences in displays of aggressive behaviours have been traced to genetic inheritance, and phenotypically different forms of aggression, which share similar functions, may also share a similar pattern of genetic inheritance (Higley 2001).

Strong links are evident between mammals and fish, by the broad location in the hypothalamus and telencephalon, of high numbers of differentially expressed genes relating to aggressiveness, and the expression of these genes relative to social rank (Filby *et al.* 2010). Furthermore, studies have shown altered, at times conflicting, neurophysiological responses based on coping styles closely linked to phenotypic behavioral types in both mammals (Miczek *et al.* 1998) and fish (Winberg *et al.* 1993) and crustaceans (Kravitz 2000). Based on work on rats and mice bred for high or low aggressiveness (Jones 1989; Miczek *et al.* 1998; Ribeiro *et al.* 2015) it has been suggested that proactive aggressive behaviour is negatively related to 5-HT neuronal activity while the inverse is apparent for reactive behaviour. In mice selectively bred for high / low aggression, high proactive aggression was negatively correlated with Tph functioning (Metcalfe 1989). In dominant, and isolate (resident) crayfish 5-HT has a facilitating effect on synaptic transmission, while in subordinates 5-HT reduced the magnitude of the synaptic response (Yeh *et al.* 1996; Yeh *et al.* 1997). It was suggested that the 5-HT mediated response was via different receptors, and interestingly the changes from the types of modulation observed in isolates, to that seen in dominants and subordinates occurred linearly over a 12 day period. Subordinate crayfish and lobsters were more likely to initiate fighting, and duration and intensity of fights was elevated in animals injected with 5-HT, however these observed responses were delayed for about 45 minutes (Kravitz 2000). When in pairs, subordinate arctic char were observed to completely cease feeding leaving the dominant fish to monopolise the food supply; furthermore, brain serotonergic activity described by 5-HIAA : 5-HT, was elevated in subordinate fish (Overli *et al.* 1998) . Studies on zebra fish have shown that genes encoding for TRP hydroxylase (Tph), the synthesizing and rate-limiting enzyme for 5-HT, and for the htr1a receptor, were overexpressed in dominant versus subordinate males (Filby *et al.* 2010). Similarly, other traits with neuroendocrinological starting points, such as responsiveness to stressors, have been shown to be heritable, and to differ both between strains, populations and individuals, with some fish displaying consistently high or low responsiveness (Bakker 1986; Pottinger *et al.* 1994; Pickering 1998; Glencross 2008).

While much research has been done on the neurological pathways associated with behavioural interactions, stress response and hypophagia, the inter-relationships between these pathways,

and many others, are extremely complex and not well understood. The overwhelming body of evidence detailing the strong role in behavioural modulation played by the serotonergic system is indisputable, as is its conservation across taxa. The use of selective serotonin re-uptake inhibitors, and serotonin receptor agonists and antagonists provides clear observation of the association between 5-HT receptor functionality and various behavioural types or histories. However 5-HT function is also implicated in depression, post-traumatic stress disorder, temperature and endocrine regulation, sleep, locomotion learning and memory, sexual function, and immune activity (Summers *et al.* 2005). This wide array of functions affected by 5-HT or 5-HT turnover, combined with observed differences in response relative to social rank, subordination or stress responsiveness, make it almost impossible to believe that modified serotonergic activity in isolation can be a moderator of aggressive behaviours. Perhaps analogously alcohol has an effect on numerous behavioural, physiological and psychological functions. Tellingly some, but not all individuals, consistently exhibit increased aggression after exposure to alcohol, and the 5-HT system has been identified as a modulator of these behaviours in this type of aggressor (van Erp and Miczek 1997; Miczek *et al.* 1998; Higley 2001).

Loosely speaking it could be suggested that increased serotonin and / or serotonergic turnover modulates aggressive behaviour in more aggressive animals, while increasing boldness in less aggressive animals. There is no reason to suppose the fish used in the current study, both residents and intruders, weren't normally distributed across a spectrum of dominance, from strongly subordinate to strongly dominant, and therefore, if this tenet were applied to the current study, one might expect a more limited variation in behavioural response from those fed a TRP supplemented feed. Reasons why this response was not observed might include (1) insufficient supplementation of TRP, (2) insufficient replication, or (3) insufficient knowledge of the drivers of aggressive behaviours and cannibalism in juvenile barramundi.

L-Tryptophan was added to the feed mix at 14 $mg.g^{-1}$ and this produced a feed containing 19.4 $mg.g^{-1}$ of total TRP. No confirmation of the increased transportation of TRP via the blood, across the blood brain barrier, and its conversion into 5-HT were taken in this experiment. Numerous studies on other species, as well as studies by the author (Chapter 2 of this thesis) confirm the

up-regulation of the serotonergic system at this level of supplementation. Furthermore, as has been described, the modulation of aggressive behaviours has been observed in other species at a similar level of inclusion, which raises the question; why would a system shown to be so evolutionarily conserved display such a wide variety of responses? Differences between responses are apparent at phylum, class, genus, and species, as well as at the individual level.

Given the extremely wide behavioural response elicited from elevated serotonergic activity, the current experiment may have been insufficiently robust to observe any changes. Nine replicate resident and intruder pairs at each day studied were considered sufficient based on the relevant literature, however cannibalism exceeded expectations thus reducing replication. This was particularly evident in the later hours of observation for each day, however did not impact data for the initial observation window, and only minimally for the 6h observation window. Whilst the loss of replicates did impact the veracity of the data for the later timepoints it should be noted that no correlation was observed between any measured aggressive behaviours and cannibalism, and nor was cannibalism different between feed treatments.

While the examination of behaviours displayed by commercially important salmonid species is extensive, there are no published data exploring behavioural displays in barramundi, apart from limited work surrounding cannibalism. Intracohort cannibalism in juvenile barramundi is reduced with increased feeding frequency, reduced light intensity, and the provision of refugia, however persists even under a twice daily satiation feeding regime (Qin *et al.* 2004; Applebaum and Arockiaraj 2010). This suggests that though hunger may exacerbate cannibalism it is not the only driver. It is not inconceivable that in fish, unlike for example humans, cannibalism as a feeding strategy may develop by accident as the result of hierarchical aggression. Interestingly cannibalism is most often excluded from discussion on aggressive behaviours. Some fish appear more likely to pursue cannibalism as a strategy than others suggesting a hereditary basis for cannibalism, bolstered by evidence of preferential bioenergetics of an exclusively cannibalistic diet over a pelleted feed diet (Ribeiro and Qin 2016). It is suggested that cannibalism in barramundi should be viewed as a feeding strategy with strong positive evolutionary outcomes,

rather than an action undertaken to maintain or elevate hierarchical stature, despite that being a probable outcome.

Barramundi are protandrous hermaphrodites and thus all interactions between juveniles are intermale by sex, if not always by behavioural definition. The most reliable measure of when a barramundi transforms from a male to a female is length, and therefore those with the fastest growth rate receive the enormous evolutionary benefit of sex change earliest, and as fecundity is a function of size, larger individuals have a proportionately greater impact on the overall stock. Despite significant evidence for a calmative and hypophagic effect of supplementary dietary TRP via increased 5-HT turnover, there was no evidence from survival analysis in the current experiment that supplementation at 14 $mg.g^{-1}$ had any effect.

The aim of experiment 2 was to identify whether patterns existed in responses to three stimuli which could be used to attribute a behavioural type to individuals, and to examine the physiological stress response and serotonergic activity of these individuals relative to that behavioural type. Unfortunately a processing problem meant the data for brain serotonin activity were unusable following machine error, however some interesting correlations between behaviours, and behaviours and physiological stress response exist.

A difference in body size of 30% between pairs was sufficient to illicit a chase response by the larger resident in 32 of the 40 pairings. Conversely, in only 3 of the 40 pairings did the smaller intruders chase the larger resident, and in only one of those pairings did the larger resident not also chase the smaller intruder. The mean time spent chasing the larger resident by the 3 smaller intruders that did, was approximately 32 seconds, while the mean time spent chasing the smaller intruder by the 32 larger residents that did was approximately 58 seconds. Similarly all bar one of the larger resident fish eyeballed the smaller intruder, and the mean time spent eyeballing was approximately 86 seconds, while the same 3 smaller intruders that chased the larger residents were the only 3 to eyeball also. Mean time spent eyeballing the larger resident by the smaller intruder was approximately 26 seconds. These findings on size dominance in juvenile barramundi pairs broadly reflect similar findings in other species where larger fish are more able to secure a greater proportion of the food (Jobling and Wandsvik 1982; Wootton

1990). In pairs of Atlantic salmon parr with a size difference of less than 5% larger fish were dominant in 54% of cases, while when the size difference was greater than 5% larger fish were dominant in 73% of cases (Huntingford *et al.* 1989) . In Arctic char of similar sizes, size was not found to significantly influence either level of aggression or amount of food acquired (Adams and Huntingford 1996). It is widely accepted however that though size assists individuals in dominating the available resources, the drivers behind size disparity are traits such as fierceness (Bakker 1986; Huntingford *et al.* 1989; Johnsson *et al.* 1996). In the current study there was a 30% difference in size between the larger and smaller fish, sufficient for cannibalism to occur, however the smaller intruder was dominant in 7.5% of cases. Dominance was expected to have been affected by the level of fierceness, previous interactions, residency, and body size of both combatants, so no firm conclusions can be made as to the precise reasons behind the overall dominance outcomes of the intruder test, however it is suggested that the size difference was a significant factor.

Surprisingly, apparently-subordinate fish in the intruder test did not necessarily display subordinate behaviours when confronted with a same size conspecific in the form of a mirror image, showing that dominance among juvenile barramundi can be highly flexible and situation dependent. Fish delivered more than twice the number of attacks per minute to their mirror image than to a smaller intruder. Interestingly the most aggressive four fish accounted for over half of the total mirror image attacks however these four fish delivered no attacks on the smaller conspecific intruder. Though counter-intuitive it is possible that the fish which exhibited extremely elevated rates of attack on their mirror images considered they had lost the bout, and in their following encounter behaved relative to their previous loss. The influence of social experience in animals is such that a previous victor is more likely to win subsequent interactions with a different opponent while a previous experience of losing increases the probability of future loss (Dugatkin 1997; Hsu and Wolf 1999; Oliveira *et al.* 2009). Alternatively, the presentation of a same-size intruder in the form of a mirror image, may have elicited a stronger hierarchical response than the smaller, actual intruder. Alternatively, the mirror image may have been viewed as competition while the intruder may have been viewed as food.

The proportion of time spent eyeballing was 31.8 %, 10.3 % and 13.5 % for the mirror, foreign object, and intruder tests respectively providing further evidence that the mirror image elicited a stronger aggressive response than the smaller intruder. Perhaps the elevated aggressive response displayed toward the mirror image was the result of a lack of subordination on the part of their reflection. Size parity may also have been a factor in more drawn out aggressive displays as, when in groups, same size individuals appear to be more regularly in combat, possibly as the need to assert dominance is stronger among these fish. Furthermore this combat is rarely between the largest or the smallest fish. Desjardins and Fernald (2010) observed vastly different gene expression in the hippocampus and amygdala, a location rich in corticotropin-releasing hormone containing neurons (Schulkin *et al.* 1998), of the African cichlid *Astatotilapia burtoni* when fighting with a mirror image, compared to when fighting with a conspecific across a clear barrier. They suggested a fear induced response may mediate the contrast as a mirror image is completely novel stimulus, not interpretable by previous combat as the mirror combatant does not react in familiar ways.

Though no association was observed between occurrence or duration of attacks between the three stimulus tests were observed, a significant regression equation was found for the occurrence of attacking the mirror, showing that blood lactate, eyeballing intruder, and ignoring intruder accounted for 43% of the variance in the occurrence of attacks. Total time spent eyeballing intruder and ignoring intruder were inversely related to the occurrence of mirror attacks. It is expected that a fish that is more likely to ignore a possible aggressive interaction might also be less likely to instigate an attack on a mirror image. A reduction in the total time spent eyeballing the intruder being associated with increased occurrence of attack on a mirror image is harder to explain. It is possible that smaller subordinate intruders submitted to eyeballing and thus attack was unnecessary, while the reflection of course did not. The strongest predictor of attacking the mirror was blood [lactate] suggesting that possibly more subordinate fish were more likely to engage with a mirror image than with a conspecific.

Contrary to what had been hoped for it appears impossible to use either the responses to mirror images or foreign objects in lieu of, or as proxies for, dyad interactions between juvenile

barramundi. Despite multiple stepwise regressions identifying significant predictors of the dependent variable, some of the results, especially those across tests are difficult to justify. Both eyeballing and facing foreign object, as well as facing mirror and facing left wall during the mirror test were significant predictors, and accounted for 52% of the variance in time spent chasing the intruder. Of these 4, time spent eyeballing foreign object was the strongest predictor, which perhaps sits most comfortably, as eyeballing foreign object appears to be the most aggressive of the predictor actions. Eyeballing mirror however is not a predictor of chasing intruder, so eyeballing per se cannot be presented as a measure associated with increased intruder chasing. Furthermore, eyeballing intruder is not a predictor for chasing intruder, indeed no independent, intruder test measures, were found to be significantly correlated with chasing intruder. Though significant predictors within the model, facing left wall and facing mirror are harder to justify in a behavioural or biological context, both allow the fish to see its reflection and thus might be more aligned with the observe intruder in the intruder test however no such correlations exist. Total time spent facing left wall in the mirror test was however a predictor of the total variance in post intruder test blood lactate, which itself was higher in residents which had been chased by intruders, and showed a negative association with the total time for eyeballing foreign object. These behaviours might be expected from the less dominant individuals rather than those that were more prone to chase the intruder.

There was a significant regression equation for ignoring intruder showing that facing right wall in the foreign object test accounted for 15.6% of the variance in the total time spent ignoring intruder. Whilst facing right wall would be perceived as a non-combative behaviour, closer in nature to ignoring than to many of the other recorded behaviours, the lack of facing left wall also being a predictor, as theoretically these two orientations are identical, casts doubt on the behavioural interplay between the predictor and variable in this model.

The lack of clear associations between the behaviours of individuals across the tested stimuli is not an isolated finding and may be a species specific response. Atlantic salmon parr that competed effectively for food in small groups when supply was limited and distribution was localized also performed well at high density when offered food *ad libitum* (Metcalfe 1989;

Metcalfe *et al.* 1989). A similar study on Arctic char however showed no ability by previously dominant fish that had monopolized the food resource in pairwise tests, to subsequently continue this behaviour in a more traditional tank dynamic.

A strong within test regression equation was found for blood glucose, as already mentioned, showing that total time spent chased by intruder accounted for 36.6% of the total variance in blood glucose. This is interesting as the total time spent being chased by the intruder was much less than the total time the intruders spent being chased by the resident, showing that the act of chasing, and carrying out other dominant, aggressive behaviours doesn't elevate blood glucose, presumably as a result of HPI axis activation, but being chased does. Total time spent being chased by the intruder was also found to account for 96.1% of the variance in total time spent being eyeballed by intruder which is expected and easy to justify; barramundi focus on their target prior to attacking or giving chase. Conflictingly, this association was not present for the opposite scenario, e.g. by residents towards intruders. Despite these fairly strong and justifiable within test regression equations, a further strong regression equation for failed attack (comparable to an attack on the mirror of foreign object) showing that 5 predictors account for 76.1% of the variance in the total number of failed attack is harder to justify. Whilst occurrence of eyeball intruder was the strongest predictor in the model and fits ambush predation techniques as previously explained, the other 4 predictors, all measures of total time, are negatively associated with the occurrence of failed attack. Possibly failed attack, which is an apparent attempt to grab the intruder, should be viewed as a purely cannibalistic behaviour more closely associated to feeding than to chasing, and the suite of aggressive actions that don't directly have cannibalism as a likely endpoint, should be viewed as hierarchical jostling. Barramundi are type 2 cannibals; ambush predators, without buccal teeth, and unable to easily take hold of or bite their prey, and thus may not be more successful cannibals by increasing their prey chasing. They may however be more successful by waiting (eyeballing) for the best moment to strike.

In conclusion there appear to be no advantages of supplementing a barramundi feed with TRP to a total inclusion of 19.4 $mg.g^{-1}$ in order to reduce aggressive behaviours. Consequently the

hypothesis: Supplementing fish food with TRP will reduce the occurrence of cannibalism by juvenile barramundi, compared to those fed a non-TRP supplemented feed, can be rejected for pairwise responses at the level of inclusion of this study. When a 50% smaller conspecific was added to a tank containing a larger resident after 1, 2, 4, 6 and 14 days of TRP supplemented feed delivery, neither the occurrence nor time spent exhibiting the observed behaviours was different from the reference feed fed fish in a way that was considered of biological significance. Furthermore, no differences in survival of the intruder over a 24 hour period were present after 1, 2, 3, 4, 5, 6, 7, 8, or 14 days. At a ration of 8% $BW.day^{-1}$ no hypophagic effect of TRP supplementation at 19.4 $mg.g^{-1}$ was observed.

Individual barramundi display individual behavioural responses as evidenced by some resident fish in Exp 2 behaving in a subordinate way toward a smaller intruder. However consistent responses from individual fish across stimuli were not observed and neither did physiological stress response provide evidence of behavioural type. It is not possible to use reactions to either a foreign object or a mirror image as proxies for behavioural interactions between a resident barramundi and a 30% smaller intruder. Given that serotonergic activity data were not useable it is concluded that the hypothesis: When presented with 3 different stimuli, juvenile barramundi will respond consistently in behaviour, endocrine stress and serotonergic response depending on their behavioural type, can be rejected.

3 Chapter 3 - The effects of tryptophan inclusion level and ration size on intracohort cannibalism, growth performance, serotonergic activity and physiological stress response in juvenile barramundi

3.1 Introduction

Cannibalism is widespread, occurring in almost all major vertebrate and invertebrate groups (Elgar and Crespi 1992). Similarly it is widespread among freshwater organisms (Spence and Carcamo 1991), common in teleost fish species (Smith and Reay 1991) many of which are of commercial importance: African catfish *Clarias gariepinus,* barramundi *Lates calcarifer,* rainbow trout *Oncorhynchus mykiss,* striped bass *Morone saxatilis*, European seabass *Dicentrarchus labrax*, chub mackerel *Scomber japonicus*, Atlantic cod *Gadus morhua*, European eel *Anguilla Anguilla*, common carp *Cyprinus carpio*, northern pike *Esox Lucius*, and Japanese amberjack *Seriola quiqueradiata*; (see review, Hecht and Pienaar 1993) . The prevalence of cannibalism among barramundi is well documented especially over larval and juvenile phases while growth rates are at their highest (MacKinnon 1985; Duray and Juario 1988; Parazo *et al.* 1991; Qin *et al.* 2004; Curnow *et al.* 2006; Appelbaum and Arockiaraj 2010; Ribeiro and Qin 2013). The potential for size-dependent cannibalism amongst individuals has been examined (Parazo *et al.* 1991; Ribeiro and Qin 2013), as have the growth performance advantages (Ribeiro and Qin 2016), and some mitigation strategies (Qin *et al.* 2004). The consequences of cannibalism, described for other teleost species are mortality, damage, disease, and social subordination. The effects of the non-fatal encounters are reduced immune function, suppressed feeding response, reduced food intake and reduced feeding efficiency (Hart 1993). Despite cannibalism requiring time-consuming management interventions in the commercial production of barramundi, and the direct cause of significant stock loss, no substantial effort has been made to understand this aspect of the species nor have many strategies been pursued to reduce the impact.

Agonistic behaviours have been examined in a range of teleost fish including fish including chinook salmon (*Oncorhynchus tshawytscha*) (Taylor and Larkin 1986), coho salmon

(*Oncorhynchus kisutch*) (Riddell and Swain 1991), Nile tilapia (*Oreochromis niloticus*) (Alvarenga and Volpato 1995), bicolour damselfish (*Pomacentru partitus*) (Winberg *et al.* 1996), Atlantic salmon (*Salmo salar*) (Damsgard and Arnesen 1998), brown trout (*Salmon trutta*) (Lahti *et al.* 2001), Amargosa river pupfish (*Cyprinodon nevadensis amargosae*) (Lema and Nevitt 2004), Zebrafish (*Danio rerio*) (Larson *et al.* 2006), European sea bass (*Dicentrarchus labrax*) (Di-Poï *et al.* 2007), and cichlid fish (*Oreochromis mossambicus*) (Oliveira *et al.* 2009). The effects of agonistic behaviour, such as the formation of dominance hierarchies, involve aggression, include elevated stress responses, and are thought to be responsible for considerable production losses in many fish culturing systems (Winberg and Nilsson 1993a). Growth depensation is exacerbated by agonistic behaviours as dominant fish further monopolise the food resource, and thus grow faster than subordinates. The ensuing size difference further entrenches dominant / subordinate relationships and the monopolization of the feed resource (Wootton 1990; Adams and Huntingford 1996). Food distribution and ration size have also been reported as factors influencing growth depensation in fish (Olla B and Samet 1974; Keast and Eadie 1985). Intracohort variability in size can favour cannibals.

There are two types of cannibalism observed in fish: Type 1 cannibalism involves cannibals taking bites from prey fish or attempting to consume them tail first, often leaving the head. Type 2 cannibalism, that observed in barramundi and many other species, involves the cannibal consuming the prey whole and head first. Type 2 cannibalism therefore, is not possible without variation in size among the population and prevalence increases with increasing growth depensation. Juvenile barramundi eat voraciously, convert ingested feed to body mass very efficiently and therefore display intra-cohort variability in size over a shorter period than many other slower growing species. The impacts of behavioural type (dominant, subordinate, risk taking, timid), and position within social and feeding hierarchies are well documented factors influencing growth performance in fish (Jobling and Wandsvik 1982; Koebele 1985; Abbott and Dill 1989; Johnsson *et al.* 1996; Sloman *et al.* 2000; Dou *et al.* 2004; Cubitt *et al.* 2008).

Elevated serotonergic activity in the brain is a moderator of agonistic behaviours (Winberg *et al.* 2001; Hoglund *et al.* 2005; Clotfelter *et al.* 2007; Filby *et al.* 2010; Leopoldo *et al.* 2010), and is

also implicated in reduced food intake and hypophagia (Winberg *et al.* 1993; de Pedro *et al.* 1998b; Ruibal *et al.* 2002; Halford *et al.* 2007; Pérez-Maceira *et al.* 2016). Experiments were designed to answer the questions: Does dose (TRP) or ration size mediate fewer agonistic interactions, weaken social and feeding hierarchies, deliver a hypophagic response favouring subordinate individuals (or those that were getting a smaller share of the food resource), and result in reduced growth depensation.

- Hypothesis: Supplementing fish food with TRP will reduce the occurrence of cannibalism by juvenile barramundi, affect growth performance, serotonergic activity, and physiological stress response, compared to those fed a non-TRP supplemented feed

Specifically these experiments aim to examine how:

- Supplementary dietary TRP affects food intake, FCR, growth rate and growth depensation of juvenile barramundi over a 50 day period;
- Supplementary dietary TRP affects brain serotonergic activity
- Supplementary dietary TRP affects rates of cannibalistic-associated mortality in juvenile barramundi over a 50 day period;
- Ration size affects rates of cannibalistic-associated mortality in juvenile barramundi over a 50 day period;
- Supplementary dietary TRP affects the physiological stress response, as indicated by blood glucose and lactate concentrations and by whole body cortisol concentrations.

3.2 Materials & Methods

Two sequential experiments (Experiments 3 & 4) using the same recirculating system were conducted to examine the effect of supplementary dietary TRP on brain TRP metabolism, physiological stress response, growth performance, and cannibalism in juvenile barramundi. Experiment 3 measured these parameters over a broad range of dietary TRP supplementation levels for fish fed to satiation, while experiment 4 examined the effect of two different rations (satiation and 50% of satiation), using feeds supplemented with TRP at levels based on growth depensation and hypophagic responses observed in experiment 3.

3.2.1 Fish and experimental system

The experiments were conducted at the IMAS Aquaculture Centre within the University of Tasmania's Launceston campus. A recirculating system was constructed comprising 24 conical based 85 L tanks, 1.5 mm plastic screens and Dacron mesh for particulate removal, biofiltration media, and UV irradiation (1 x 40W Emperor Aquatic, Pottstown, PA, USA) for bacteriological control. Water temperature was maintained at 30°C by an immersion heater (Helios Temperature Controller, CAT HC 2/0 – 40, Australia), and maintenance of ambient air temperature in the room housing the tank system by a reverse cycle air conditioning unit (Mitsubishi Electric, MSZ-GE80VA, Australia). An Onga 413 pump delivered a directional flow of 3.5 $L.m^{-1}$ of fresh water to each tank giving a 2.5 fold turnover of tank volume each hour and sufficient centripetal force to deliver faecal waste and uneaten feed to the centre of the tank within 10 s. Effluent water was extracted from the centre of the tank base at 33 $cm.s^{-1}$ via a 15 mm diameter PVC pipe which passed through the tank wall 5 cm below the top of the tank. By this method uneaten feed was extracted from the tank within 1 minute of settling. Tanks were fitted with a 20cm rigid mesh collar which allowed for complete light penetration and ease of observation and feeding however prevented aerial escape. Aeration was provided using airstones attached to the effluent pipe but a short distance from the end of the outlet pipe to avoid disturbing pellet extraction. Photoperiod was maintained throughout the experiments at 12:12 L:D (lights on 09:00 h at 150 lux) similar to ambient photoperiod across the geographic range for the species. Throughout the experiments water from the municipal supply, treated

with sodium thiosulphate to neutralise chlorine, was added at an approximate rate of 10% of total system volume per day.

Juvenile barramundi, sourced from West Beach Aquaculture, South Australia, were air freighted to Tasmania using standard live fish air freight protocols and were transferred by road from Launceston Airport to the IMAS Aquaculture Centre. Fish were transferred to one 85 L holding tank at 30°C for transport recovery prior to randomized distribution in the experimental system. During the experiment fish were fed twice daily, in the morning between 08:00 and 10:00 h and in the afternoon between 17:00 and 19:00 h. Feeding was conducted by hand.

In experiment 1 each tank was visited twice during each feeding session with fish being fed until they ceased to ingest feed and waste pellets were observed on the screens. In experiment 2 fish were fed half of their daily ration at each feeding time. After each round of feeding, uneaten feed was collected. Food intake was calculated as *feed ration – feed waste = food intake (g.day^{-1})*, and presented as daily consumption as a percentage of body weight. Behavioural and gross health observations were made at feeding time, and dead and moribund fish were removed and measured for total length and weight. Every 10 days commencing day 5 all fish were removed from tanks, lightly sedated (15.0 mg.L^{-1} AQUI-S, New Zealand) collectively weighed in a known volume of water to the nearest mg and counted to monitor mean weights and cannibalism. Every 10 days commencing day 10 fish were handled in the described manner, counted, weighed individually to the nearest mg and measured for total length to the nearest mm. These data were used to provide accurate growth performance measures. On day 50 fish were anaesthetised (18.0 mg.L^{-1} AQUI-S) and five from each tank were randomly selected, measured for length to the nearest mm and weight to the nearest mg, and immediately sampled with handheld devices for blood glucose (ACCU-CHEK Performa Nano, Roche Diagnostics, Germany), and lactate (Lactate Pro, ARKRAY Inc. Japan), prior to euthanasia via spinal transection. The brain was then expeditiously removed by opening the cranium with a downward scalpel incision toward the mouth and immersed in liquid nitrogen. The removal and freezing of the brain took less than 90 seconds. Brains were transferred to -80°C storage until

they were prepared for analyte quantitation. The remaining fish from each tank were measured for length, to the nearest mm, and weighed to the nearest mg.

3.2.2 Feeds and rations - Experiment 3

Eight experimental feeds (1 reference and 7 with supplementary TRP – Table 3.1, p 84) were made by hammer milling an appropriate commercial feed (Nova ME, 3mm, Skretting, Cambridge, Tasmania). The resultant meal was then re-pelleted through 2 and 3 mm dies after adding the required amount of TRP, balancing the supplementary TRP with α-cellulose and using carboxy methyl cellulose (CMC) as a binding agent. See Table 3.1 (p 84). Both 2 and 3 mm pellets were produced to provide appropriately sized feed for fish as they grew.

On the 26th of October 2014 each experimental tank was stocked with 25 fish with an average length and weight of 30.12 ± 0.06 mm and 0.331 ± 0.003g respectively and all fish were observed to be feeding and behaving normally. Experimental feeds were introduced to the fish the following day and thus the 27th of October was considered day 1. The eight feeds were delivered in triplicate across 24 tanks.

3.2.3 Feeds and rations - Experiment 4

Experiment 4 utilised four feeds (1 reference and 3 with supplementary TRP –Table 3.2, p 85). The reference feed was the same as that used in experiment 1, while the feed with the most supplementary TRP, feed 4 (19 mg.Kg^{-1}), was the same feed as the one with the least TRP (feed 2) in experiment 1. Two further feeds were developed using the previously described techniques and commercial feed, with TRP supplementation at 14.8 mg.Kg^{-1} and 15.9 mg.Kg^{-1}. See Table 3.2 (p 85). Previous studies by the author had shown no impact on food intake in juvenile barramundi presented with TRP supplemented (15.3 mg.Kg^{-1} TRP) feed when fed a ration of 8 % BW.day^{-1}. Each feed was delivered at two ration levels. A linear regression of average fish weight vs food intake as a percentage of body weight delivered was produced from fish fed feed 1 and feed 2 to satiation in experiment 1. Resultant equations were used to calculate a satiation ration for fish fed feed 2 from experiment 3 (ration 1) which was delivered at all TRP inclusions, and a 50% satiation ration for each of the 4 feeds in experiment 4 (ration

2) based on average weight of fish every 5 days and observed FCR data. The same protocol was used to determine ration size for feeds 2 and 3 based on data from sample fish. The four feeds at two rations were delivered in triplicate across 24 tanks.

On the 15th January 2015 each experimental tank was stocked with 25 fish with an average length and weight of 27.43 ± 0.04 mm and 0.264 ± 0.001 g respectively and all fish were observed to be feeding and behaving normally. Experimental feeds were introduced to the fish the following day and thus the 16th of January was considered day 1.

Table 3.1 Measured composition of feeds from experiment 1 delivered to juvenile barramundi throughout a 50 day period. Feeds were created using a Skretting barramundi feed (*Nova ME 3mm) as a base with supplementary tryptophan, balanced with α-Cellulose and CMC as a binder. The mix was extruded through 2 mm and 3mm dies. (n/a = not available)

Ingredient composition (mg.g^{-1}) DM	Commercial feed*	Feed 1	Feed 2	Feed 3	Feed 4	Feed 5	Feed 6	Feed 7	Feed 8
Base commercial feed	1000	938	938	938	938	938	938	938.2	938.2
CMC	n/a	20	20	20	20	20	20	19.87	19.87
α-Cellulose	n/a	42	24.5	21	17.5	14	9.5	6.89	3.59
Tryptophan	n/a	0.00	17.50	21.00	24.50	28.00	32.50	35.04	38.62
TOTAL	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.3
Water (mL Kg^{-1})	n/a	133.3	133.3	133.3	133.3	133.3	133.3	133.3	133.3
Pellet weight (mg)									
Small 2 mm		5.2 ± 0.04	8.7 ± 0.02	8.2 ± 0.08	8.1 ± 0.06	7.1 ± 0.05	5.2 ± 0.09	5.6 ± 0.04	5.1 ± 0.05
Large 2 mm		8.7 ± 0.05							
3 mm		20.5 ± 0.08	20.1 ± 0.07						
Liquid hydrolysis + UPLC (mg.g-1) DM									
Tryptophan	5	4.6	19	21.4	23.8	28	31	33.6	40.9
Chemical composition (%)									
Crude protein (%)	48.0	44.9	48.6	46.8	48.0	47.9	48.7	49.5	49.6
Crude lipid (%)	17.9	17.2	17.0	16.4	17.2	16.5	16.2	17.4	17.0
Moisture (%)	7.7	8.9	5	8.4	7.3	7.0	7.1	6.4	6.8
Ash content (%)	8.4	7.8	7.9	7.4	8.2	8.1	7.7	7.9	7.5

Table 3.2 Measured composition of feeds from experiment 2 delivered to juvenile barramundi throughout a 50 day period. Feeds were created using a Skretting barramundi feed (*Nova ME 3mm) as a base with supplementary TRP, balanced with α-Cellulose and CMC as a binder. The mix was extruded through 2 mm and 3mm dies. (n/a = not available)

Ingredient composition (mg.g^{-1}) DM	Commercial feed*	Feed 1	Feed 2	Feed 3	Feed 4
Base commercial feed	1000	938	938	938	938
CMC	n/a	20	20	20	20
α-Cellulose	n/a	42	28	26.25	24.5
Tryptophan	n/a	0.00	14.00	15.75	17.50
TOTAL	1000	1000.0	1000.0	1000.0	1000.0
Water (mL Kg^{-1})	n/a	195	200	190	137.5
Pellet weight (mg)					
Small 2 mm		4.9 ± 0.04	3.7 ± 0.02	3.1 ± 0.08	3.4 ± 0.02
Large 2 mm		5.6 ± 0.08	5.7 ± 0.06	6.1 ± 0.08	5.9 ± 0.09
3 mm		19.4 ± 0.09	20.8 ± 0.05	20.1 ± 0.06	21.9 ± 0.03
Liquid hydrolysis + UPLC (mg.g-1) DM					
Tryptophan	5	4.6	14.8	15.9	19
Chemical composition (%)					
Crude protein (%)	48.0	44.9	48.6	46.8	48.0
Crude lipid (%)	17.9	17.2	17.0	16.4	17.2
Moisture (%)	7.7	8.9	5	8.4	7.3
Ash content (%)	8.4	7.8	7.7	7.6	8.2

3.2.4 Physiological stress response

Fish were blood sampled via caudal excision for glucose and lactate concentrations as previously described. Insufficient blood was available for cortisol quantitation and therefore a whole body cortisol analysis was developed based on an ethyl ether extraction method presented by Sink et al. (2007), but adapted (method described below) to account for the range in fish size in the current experiment. Cortisol quantitation was carried out for fish fed the reference feed, and feeds 2 (lowest supplementary TRP inclusion), 4 (mid supplementary TRP inclusion) and 8 (highest supplementary TRP inclusion) from experiment 3, and for fish fed the reference feed, and feed 4 (highest supplementary TRP inclusion) at both rations from

experiment 4. In both instances nine fish per treatment (3 per replicate) were sampled. Each fish had dorsal spines and opercula removed prior to being sliced laterally into 5mm sections, and placed in a stomacher bag, weighed to the nearest mg and an equal volume of phosphate buffer saline (PBS) added. The samples were stomached for six minutes after which 1 mL of the resultant solution was removed to a 2 mL Eppendorf tube and 1 mL of ethyl ether added. After vortexing for one minute samples were centrifuged at 3500 RPM for ten minutes prior to freezing for two hours at -80° or until the aqueous layer was frozen. The upper ethyl ether layer was then extracted via Pasteur pipette to an HPLC vial which was evaporated overnight prior to storage at -80°C until ELISA were conducted on the samples. Serum cortisol was determined via an enzyme immunoassay (EIA) kit (Cortisol EIA Kit, Catalog No. ADI-900-071, Enzo Life Sciences) which is a competitive immunoassay for the quantitative determination of cortisol in biological fluids.

3.2.5 Brain tissue sampling

3.2.5.1 Quantitation

A novel method for quantitation of the desired analytes from fish brains based on a method for brain neuroendocrine determination in rat brain (Park *et al.* 2013) was developed in conjunction with David Nichols of Central Science Laboratory, UTAS. A stable isotope dilution was used for quantitation of TRP and neuroendocrine analytes 5HT and 5OH-IAA, with the addition of deuterated surrogate standards to excised brain tissue prior to homogenization and treatment. To further stabilize the sample and improve detection a derivatisation was used. This simple robust step could proceed rapidly at room temperature in the aqueous sample matrix, and the resultant ethyl chloroformate derivatives could be extracted from the aqueous mixture by organic solvent, thereby providing an additional purification step from the sample matrix. Ultra performance liquid chromatography (LC) coupled with triple quadrupole tandem mass spectrometry (MS/MS) for detection and quantitation were experimentally determined

and optimised using analytical standards of both native analytes and deuterated labelled compounds. Detection sensitivity was determined to be approximately 1pg.

3.2.5.2 Brain sample preparation

Each fish brain was removed from -80° C storage and quickly weighed to the nearest µg in a 2 mL Eppendorf tube. The tube was immersed in ice and 100 µL of the spiking standard of 2µg.mL^{-1} each of deuterated TRP, 5HT and 5OH-IAA was added. Sixty seconds later a fourfold volume of ice cold 0.1 M H_2SO_4 was added and the contents were sonicated using a Microson Ultrasonic Cell Disruptor (Microsonix Inc., Farmingdale, NY, USA) for 5 seconds at 5W. Homogenates were centrifuged (Eppendorf Centrifuge 5810R) for 15 minutes in a chilled centrifuge (4°C) at 16,600g. Supernatant was aspirated, the residue discarded and identical centrifugation was repeated. Supernatant was transferred to a 0.5 mL Eppendorf tube and stored at -80°C for derivatisation.

3.2.5.3 Derivatisation

A volume of 185 µL of thawed, ice cold brain homogenate solution was transferred to an 8 mL glass reaction vial (Cat # 98008, Grace Discovery) immersed in ice. To the vial was added 265 µL of ice cold distilled water, 300 µL of a 4:1 ethanol:pyridine solution and 20 µL of ethyl chloroformate before capping the vial and shaking for 4 minutes. To this was added 1000 µL of diethyl ether prior to freezing for 2 hours at -80^{o}C or until the aqueous layer was frozen. After removing from -80^{o}C a Pasteur pipette was used to remove the upper organic layer to an HPLC vial (Cat # 95191 Grace Discovery) and stored at -80^{o}C in preparation for HPLC.

3.2.5.4 Brain analyte quantitation

The derivatised samples were analyzed using a Waters Acquity H-Class Ultra Pressure Liquid Chromatography (UPLC) instrument coupled to a Waters Xevo triple quadrupole mass spectrometer. A Waters Acquity UPLC BEH C_{18} column (2.1 mm × 100 mm × 1.7 µm) was used.

The mobile phase consisted of two solvents: 1% (v/v) formic acid in water (solvent A) and acetonitrile (solvent B). The UPLC program was 97% A:3% B to 100% B at 6.0 min, which was held for 1 min, and this was followed by immediate re-equilibration to starting conditions for 3 min. The flow rate was 0.30 mL min^{-1}, the column was held at 35°C, and the sample compartment at 6°C. Injection volume was 1 uL.

The mass spectrometer was operated in positive ion electrospray mode with a needle voltage of 2.9 kV, and multiple reaction monitoring (MRM) was used to detect all analytes. Dwell time for each MRM transition was 18 msec. Cone voltages and collision energies were optimized for each MRM transition. The ion source temperature was 130°C, the desolvation gas was N_2 at 950 L h^{-1}, the cone gas flow was 100 L h^{-1}, and the desolvation temperature was 450°C. Data were processed using MassLynx software.

3.2.6 Calculations

Feed conversion ratio (FCR) was calculated as:

$$FCR\ (g.g^{-1}) = \text{Weight of ingested feed (g)} / \text{Weight gain of fish (g)}$$

Specific growth rate (SGR) was calculated as:

$$SGR\ (\%\ d^{-1}) = 100\ ((\ln W_t - \ln W_i)/t)$$

where W_i and W_t are initial and final fish weights respectively and t is time (days) between initial and final weighing.

The coefficient of variation (CV) was calculated as:

$$CV\ (\%) = 100\ (\text{standard deviation} / \text{mean})$$

3.2.7 Statistical analysis

Statistical analyses were performed using SPSS version 21 (SPSS, 2014) and GraphPad Prism version 6.0 for Windows, GraphPad Software, La Jolla California USA. Mean values are reported ± standard error of the mean (SEM). All distributions were found to be normal by the D'Agostino & Pearson omnibus normality test. Homogeneity of variances was tested graphically by examination of residual plots in SPSS and by the Brown-Forsythe test. Data were tested for differences between treatments using one way and two way ANOVA, or independent samples *t*-tests and multiple comparisons were made using Tukey and Bonferonni tests. Differences were considered significant at $p < 0.05$.

3.3 Results – Experiment 3

3.3.1 Food intake and growth performance

No differences were observed between treatments at the start of experiment 3 for either weight or length. See Table 3.3 (p 94). Both weight and length were affected negatively in a dose dependent manner when compared to TRP content of the diet with ANOVA showing respective differences: ($F = 410$, df 7, 404; $p < 0.0001$) and ($F = 529$, df 7, 404; $p < 0.0001$). Mean overall mortality across all feed treatments was 33.7 %; this figure included cannibalism-associated mortality, excluded moribund fish which did not consume any feed, and fish which died but not thought to be as the result of attempted cannibalism.. Differences in mean overall mortality between feed treatments were observed (Chi sq = 26.65, *df* 7, $p < 0.001$). Mean cannibalism-associated mortality across all treatments was 12.5 %. No differences in cannibalism-associated mortality between feed treatments were observed. See Table 3.3 (p 94) & Figure 3.11 (p 105).

Linear regressions identified strong correlations for (log transformed) wet weight (WW) and total length (TL) ($p < 0.0001$) (Figure 3.1, A, p 95); body depth (BD) and total length ($p < 0.0001$) (Figure 3.1, B, p 95); horizontal mouth gape (HG) and total length ($p < 0.0001$) (Figure 3.1, C, p 95); vertical mouth gape (VG) and total length ($p < 0.0001$) (Figure 3.1, D, p 95), horizontal mouth gape and body depth ($p < 0.0001$) (Figure 3.1, E, p 95); and vertical mouth gape and body depth ($p < 0.0001$) (Figure 3.1, F, p 95).

No differences were observed in food intake, measured as $g.fish^{-1}.day^{-}1$, on day 1 of experiment 3. See Figure 3.2 (p 96). By day 10 food intake was negatively affected in a dose dependent manner relative to TRP inclusion ($F = 115$, *df* 7, 16; $p < 0.0001$). From day 10 onwards food intake of fish offered feed 1 (reference feed, 4.6 $mg.g^{-1}$) was greater than that for any other feed. See Figure 3.2 (p 96). A reduction in food intake by fish fed feed 3 (21.4 $mg.g^{-1}$) compared to fish fed feed 2 (19.0 $mg.g^{-1}$) was apparent at all time points except day 1 , where no differences between any feeds were observed, and day 20, when food intake of feeds 2 and 3 were statistically similar. See Figure 3.2 (p 96). At all other time points food intake of feed 2 was

different ($p < 0.05$) to the food intake of all other feeds. The hypophagic effect of supplementary dietary TRP continued throughout the experiment and dose dependent food intake was observed on days 20, 30, 40 and 50 however differences between feeds 3, (21.4 $mg.g^{-1}$ TRP), 4 (23.8 $mg.g^{-1}$ TRP), 5 (28.0 $mg.g^{-1}$ TRP), 6 (31.0 $mg.g^{-1}$ TRP), 7 (33.6 $mg.g^{-1}$ TRP) and 8 (40.9 $mg.g^{-1}$ TRP) were not always evident and on the final day of the experiment no differences were recorded.

When juvenile barramundi were offered food to satiation twice per day containing supplementary TRP with inclusion ranging from 4.6 $mg.g^{-1}$ to 40.9 $mg.g^{-1}$, intake between treatments was not affected on day 1. Fish were fed in the morning between 08:00 and 10:00 h and in the evening between 17:00 and 19:00 h. Fish fed feed 1 (4.6 $mg.g^{-1}$ TRP) consumed more feed during the day 2 morning feed than fish fed feed containing 28.0 $mg.g^{-1}$ TRP (feed 5) or more, however no differences were recorded between fish fed 23.8 $mg.g^{-1}$ (feed 4) of TRP or less. At the afternoon mealtime on day 2 fish fed feed 1 consumed more food than fish fed any other diet except feed 2 (19.0 $mg.g^{-1}$ TRP). From the morning mealtime on day 5 food intake of fish fed feed 1 (4.6 $mg.g^{-1}$ TRP) was greater than for all feeds, including feed 2 (19.0 $mg.g^{-1}$ TRP), and remained higher for the duration of the experiment. A negative food intake response is apparent between 26 and 33 h post initial exposure for juvenile barramundi fed feed containing 21.4 $mg.g^{-1}$ (feed 3) and 23.8 $mg.g^{-1}$ (feed 4) of TRP, and between 83 and 96 h for fish fed feed containing 19.0 $mg.g^{-1}$ (feed 2) of TRP.

When food intake was analysed relative to body weight over the 50 day experiment differences ($F = 2.70$, *df* 7, 72, $p < 0.05$) were present only between the reference feed (feed 1, 4.6 $mg.g^{-1}$ TRP) and feeds 7 (33.6 $mg.g^{-1}$ TRP) and 8 (40.9 $mg.g^{-1}$ TRP) (Figure 3.3, p 97). A linear regression of food intake (Figure 3.4, p 98), measured as $\%BW.day^{-1}$, on wet weight (g) over the duration of the experiment showed highly significant differences between the y intercepts ($F = 29.19$, *df* 7, 113, p , 0.0001) indicating differences in food intake between treatments.

The daily ingestion of TRP reported as mg consumed per gram of wet weight of fish reduced over time in the reference group ($F = 41.1$, *df* 10, 22, $p < 0.0001$) (Figure 3.5, p 99), in line with the expected and observed reduction in food intake as a percentage of BW as they increase in

size over this size range. On day one extremely elevated TRP ingestion values were recorded for all TRP supplemented feeds when compared to either the control or later values. Differences in daily TRP ingestion between TRP supplemented treatments were observed at day 1 and days 25 through 50, however no such differences between supplemented feeds were observed on days 5 through 20.

More efficient feed conversion ratios (FCR) were observed for feed 1 (reference feed, 4.6 $mg.g^{-1}$ TRP) and feed 2 (19.0 $mg.g^{-1}$ TRP) when compared to all other feeds when assessed over the full 50 days of the experiment. See Figure 3.6 (p 100). When FCR was assessed at 10 day intervals throughout experiment 1, it was found to be higher in a dose dependent manner when compared to TRP content of feed ($F = 6.04$, *df* 7, 16, $p < 0.01$) despite significant differences of FCR's between feed treatments only being present at day 30. See Figure 3.7 (p 101).

Specific growth rate ($\%.d^{-1}$) was found to be negatively correlated with dosage when compared to TRP content of feed (F 42.8, *df* 7, 16, $p < 0.0001$) (Figure 3.8, p 102).

3.3.2 Endocrine stress response

No differences in tested physiological stress response parameters (cortisol, glucose and lactate) were observed between feed treatments. See Figure 3.9 (p 103).

3.3.3 Neuroendocrine response

Brain TRP ($\mu g.g\ brain^{-1}$) was found to be different when compared to TRP content of the feed ($F = 4.88$, *df* 7, 110, $p < 0.0001$) however differences between feed treatments were restricted to a difference between feed 1 (reference feed, 4.6 $mg.g^{-1}$ TRP) and all other feeds. See Figure 3.10 (p 104). There were no differences in brain serotonin ($ng.gbrain^{-1}$) between feeds. See Figure 3.10 (p 104). Brain 5OH-IAA ($ng.gbrain^{-1}$) was found to increase in a dose dependent manner when compared to the TRP content of the feed ($F = 7.67$, *df* 7, 112, $p < 0.0001$) (Figure 3.10, p

104). The ratio of 5OH-IAA: 5HT in the brain was also found to increase in a dose dependent manner when compared to the TRP content of the feed ($F = 9.45$, *df* 7, 112, $p < 0.0001$) (Figure 3.10, p 104).

3.3.4 Survival

No trend was observed when cannibalistic associated mortality was compared to dietary TRP supplementation (Logrank test for trend). A very small difference between the curves (Figure 3.11, p 105) (Chi sq = 6.867, *df* 1, $p < 0.05$) was found to be between the reference feed (feed 1, 4.6 $mg.g^{-1}$ TRP) and feed 2 (19.0 $mg.g^{-1}$ TRP), ($p = 0.007$) (Log-rank Mantel-Cox test) after p value adjustment to 0.007 (Bonferroni) was made.

Table 3.3 Performance (mean ± SEM) of juvenile barramundi fed to satiation with a pelleted feed containing varying levels of supplementary TRP throughout a 50 day period. Difference between growth performance means are identified by differing letters (Tukey's multiple comparison test, $p < 0.05$). No differences were observed between treatments for survival relative to cannibalism (Kaplan Meier with Log-rank Mantel-Cox Test).

	Weight (g)		**Length (mm)**		**Food intake (g)**	**Survival (%)**	**Mortality**	
	Initial	Final	Initial	Final	Total	Overall	Cannibalism	Other
Feed 1 (4.6 mg.g^{-1} TRP)	0.34 ± 0.0078 $^{N=75}$	29.02 $^{(f)}$ ± 1.00 $^{N=59}$	30.3 ± 0.185 $^{N=75}$	124 $^{(f)}$ ± 1.38 $^{N=59}$	1736 $^{(d)}$	78.6	16	5.4
Feed 2 (19.0 mg.g^{-1} TRP)	0.32 ± 0.0057 $^{N=75}$	12.08 $^{(e)}$ ± 0.45 $^{N=69}$	30 ± 0.164 $^{N=75}$	92.1 $^{(e)}$ ± 1.27 $^{N=69}$	790.1 $^{(c)}$	92.0	2.6	5.4
Feed 3 (21.4 mg.g^{-1} TRP)	0.32 ± 0.0064 $^{N=75}$	7.58 $^{(d)}$ ± 0.20 $^{N=55}$	30.1 ± 0.152 $^{N=75}$	79 $^{(d)}$ ± 0.791 $^{N=55}$	397.5 $^{(b)}$	73.3	9.3	17.4
Feed 4 (23.8 mg.g^{-1} TRP)	0.33 ± 0.0081 $^{N=75}$	5.13 $^{(c)}$ ± 0.22 $^{N=48}$	30.1 ± 0.181 $^{N=75}$	68.4 $^{(c)}$ ± 1.38 $^{N=48}$	292.5 $^{(ab)}$	64.0	18.6	17.4
Feed 5 (28.0 mg.g^{-1} TRP)	0.34 ± 0.0068 $^{N=75}$	4.06 $^{(bc)}$ ± 0.17 $^{N=50}$	30.2 ± 0.186 $^{N=75}$	63.1 $^{(b)}$ ± 0.918 $^{N=50}$	247.2 $^{(ab)}$	66.6	17.3	16.1
Feed 6 (31.0 mg.g^{-1} TRP)	0.33 ± 0.0077 $^{N=75}$	2.31 $^{(ab)}$ ± 0.096 $^{N=31}$	30.2 ± 0.190 $^{N=75}$	52.6 $^{(a)}$ ± 0.787 $^{N=31}$	143.5 $^{(a)}$	41.3	16	42.7
Feed 7 (33.6 mg.g^{-1} TRP)	0.34 ± 0.0079 $^{N=75}$	1.73 $^{(a)}$ ± 0.098 $^{N=44}$	30.3 ± 0.194 $^{N=75}$	48.9 $^{(a)}$ ± 1.46 $^{N=44}$	112.4 $^{(a)}$	58.6	10.6	30.8
Feed 8 (40.9 mg.g^{-1} TRP)	0.32 ± 0.0065 $^{N=75}$	1.49 $^{(a)}$ ± 0.065 $^{N=42}$	29.8 ± 0.156 $^{N=75}$	44.4 $^{(a)}$ ± 0.882 $^{N=42}$	90.57 $^{(a)}$	56.0	9.3	34.7
F	1.3	410	0.766	529	138	x^2 26.65	x^2 16.79	x^2 21.24
df	7, 592	7, 404	7, 592	7, 404	7, 16	7	7	7
p	0.259	< 0.0001	0.616	< 0.0001	< 0.0001	< 0.001	< 0.05	< 0.005

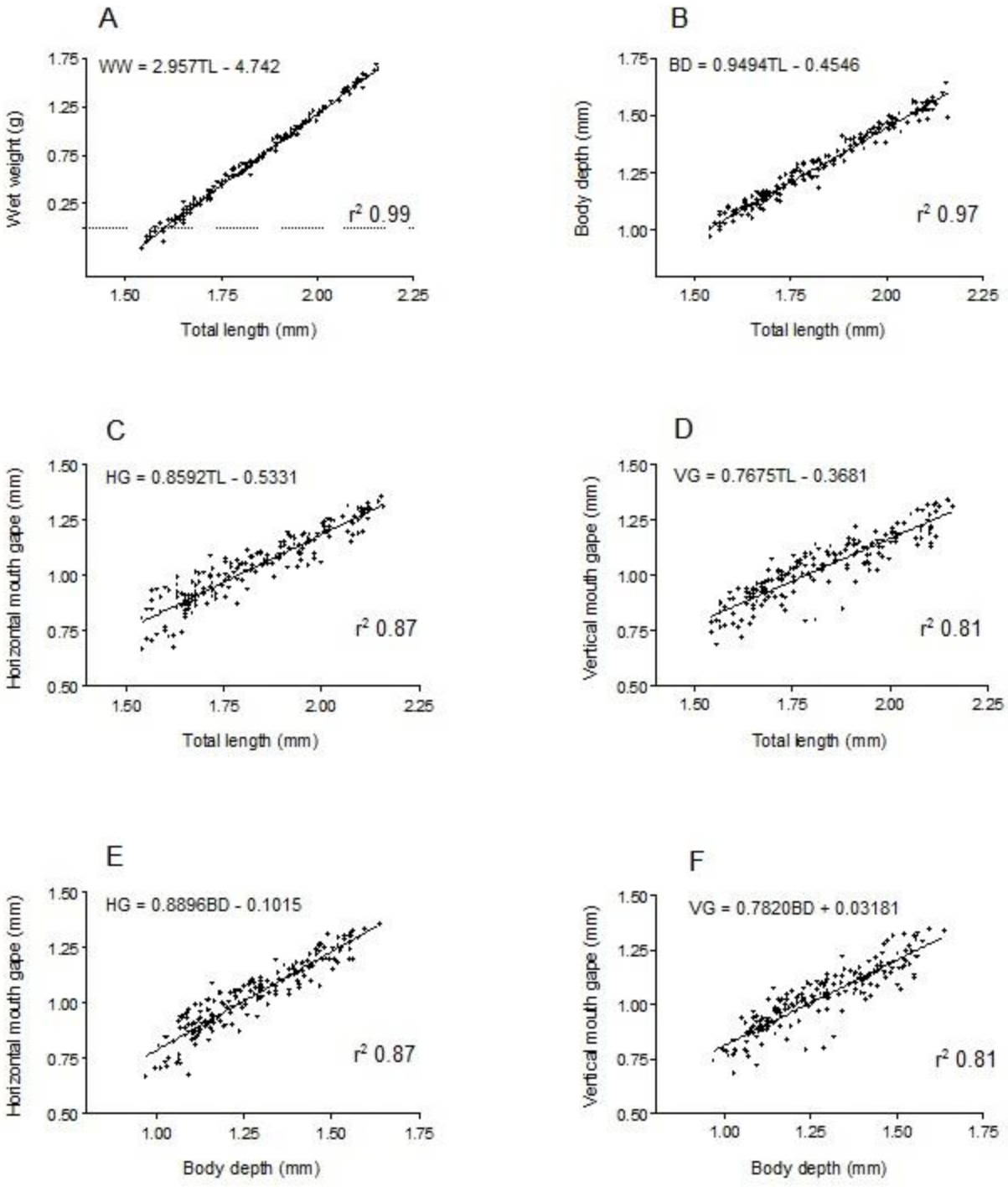

Figure 3.1 Linear regressions of log transformed size measurements of juvenile barramundi. A, wet weight (WW) and total length (TL) ($p < 0.0001$); B, body depth (BD) and total length ($p < 0.0001$); C, horizontal mouth gape (HG) and total length ($p < 0.0001$); D, vertical mouth gape (VG) and total length ($p < 0.0001$); E, horizontal mouth gape and body depth ($p < 0.0001$); and F, vertical mouth gape and body depth ($p < 0.0001$) in juvenile barramundi.

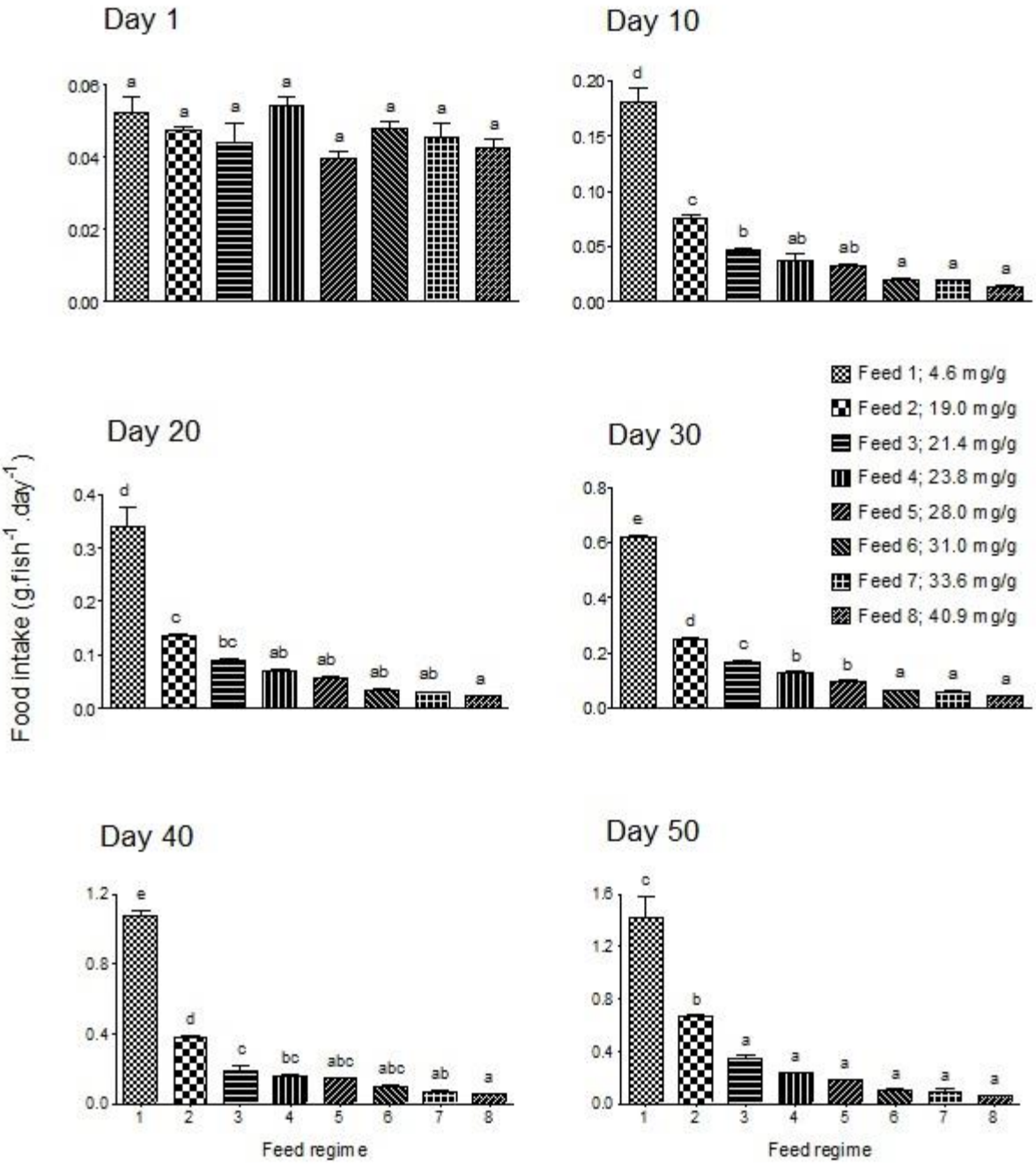

Figure 3.2 Mean daily food intake ($g.fish^{-1}.day^{-1}$) ± SEM (n=3) at 10 day intervals of juvenile barramundi fed to satiation with one of eight experimental feeds containing supplementary TRP at inclusion rates displayed in the legend as $mg.g^{-1}$ dry weight and presented as $g.fish^{-1}.day^{-1}$ at 6 time points during the 50 day experiment. ANOVA showed significant difference at day 10 ($F = 115$, *df* 7, 16, $p < 0.0001$), day 20 ($F = 60.3$, *df* 7, 16, $p < 0.0001$), day 30 ($F = 1090$, *df* 7, 16, $p < 0.0001$), day 40 ($F = 292$, *df* 7, 16, $p < 0.0001$), and day 50 ($F = 54.4$, *df* 7, 16, $p < 0.0001$). Differences in food intake between treatment at each time point are displayed by differing superscript letters (Tukey's multiple comparison test $p < 0.05$).

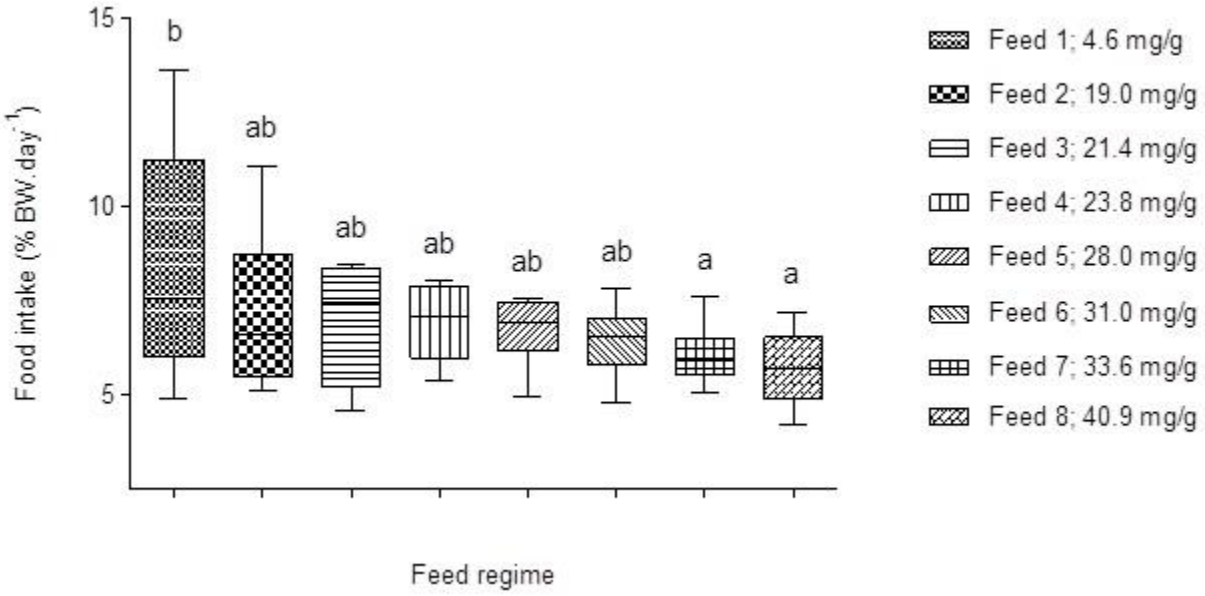

Figure 3.3 Daily food intake (% BW.day^{-1}) (n=30) of juvenile barramundi fed to satiation with one of eight experimental feeds containing supplementary TRP at inclusion rates displayed in the legend as mg.g^{-1} dry weight and presented as percent of body weight (BW).day^{-1} calculated at 5 day intervals, excluding day 1 data (no differences between treatment were observed at day 1 and thus the effect of dietary TRP is not present at this stage), during the 50 day experiment. Values shown for each feed are minimum, 25th percentile, median, 75th percentile, and maximum. ANOVA showed difference between feed treatments ($F = 2.70$, *df* 7, 72, $p < 0.05$). Differences in food intake between feed treatments are described by superscript letters (Tukey's multiple comparison test $p < 0.05$).

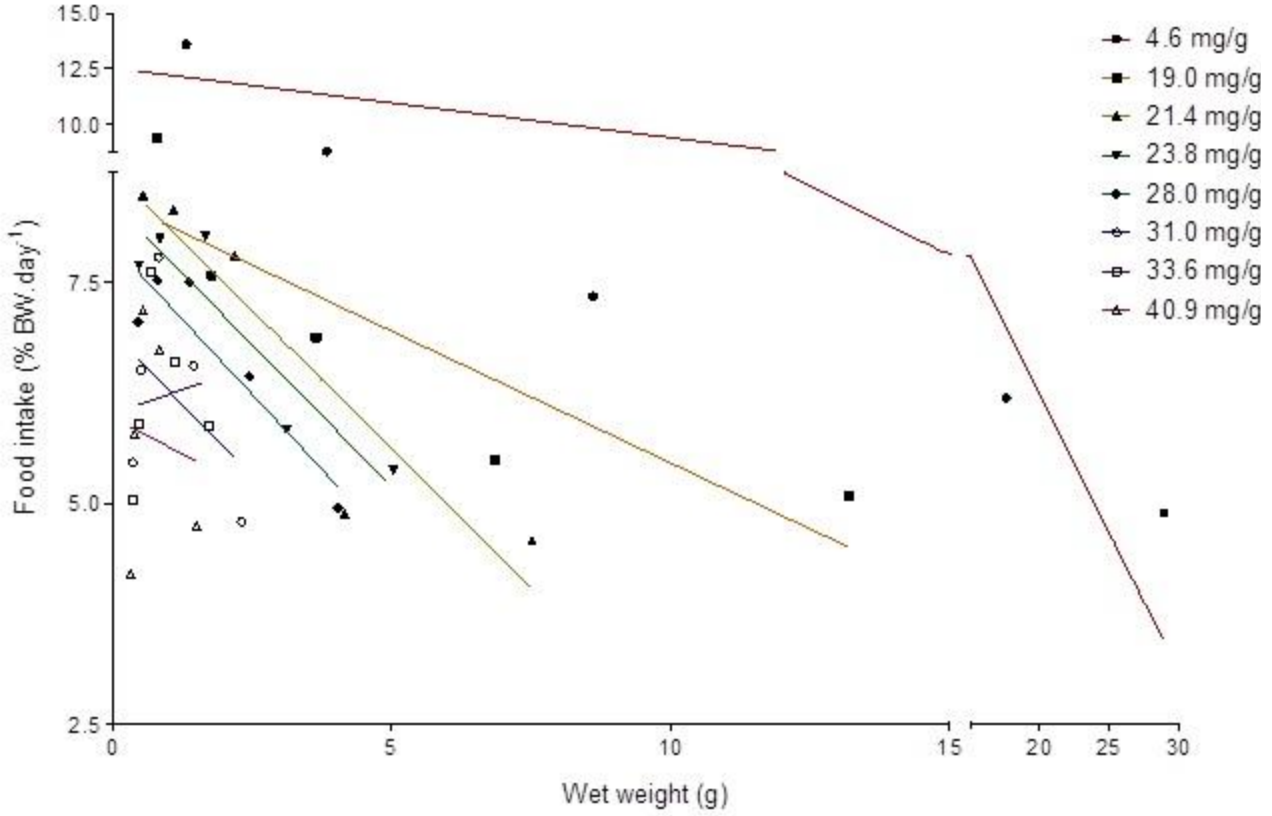

Figure 3.4 Linear regressions of food intake (% $BW.day^{-1}$) on wet weight (g) for each of the presented feeds over the course of the 50 day experiment. Data from day 1 are excluded (no differences between treatment were observed at day 1 and thus the effect of dietary TRP is not present at this stage). Negative slopes were observed for all feeds except feed 7. Regression analysis showed differences between the Y intercepts ($F = 29.19$, *df* 7, 113, p , 0.0001).

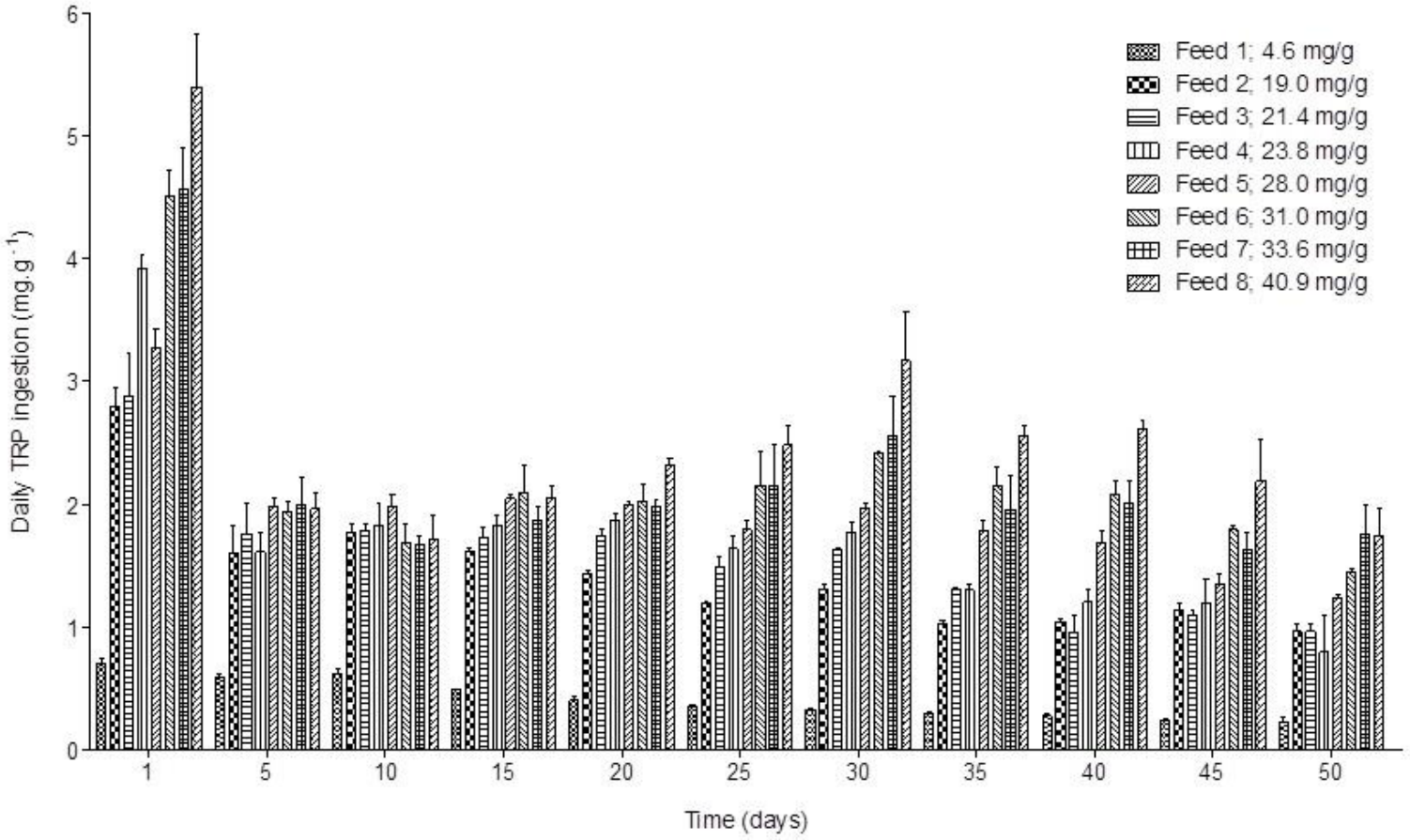

Figure 3.5 Daily consumption of TRP per gram of wet weight of juvenile barramundi fed to satiation with feeds containing supplementary TRP at inclusions shown in the legend at 11 time points during a 50 day experiment. Data are derived from daily food intake of fish per tank, and average weights measured within each 5 day interval, and are presented as mean ± SEM.

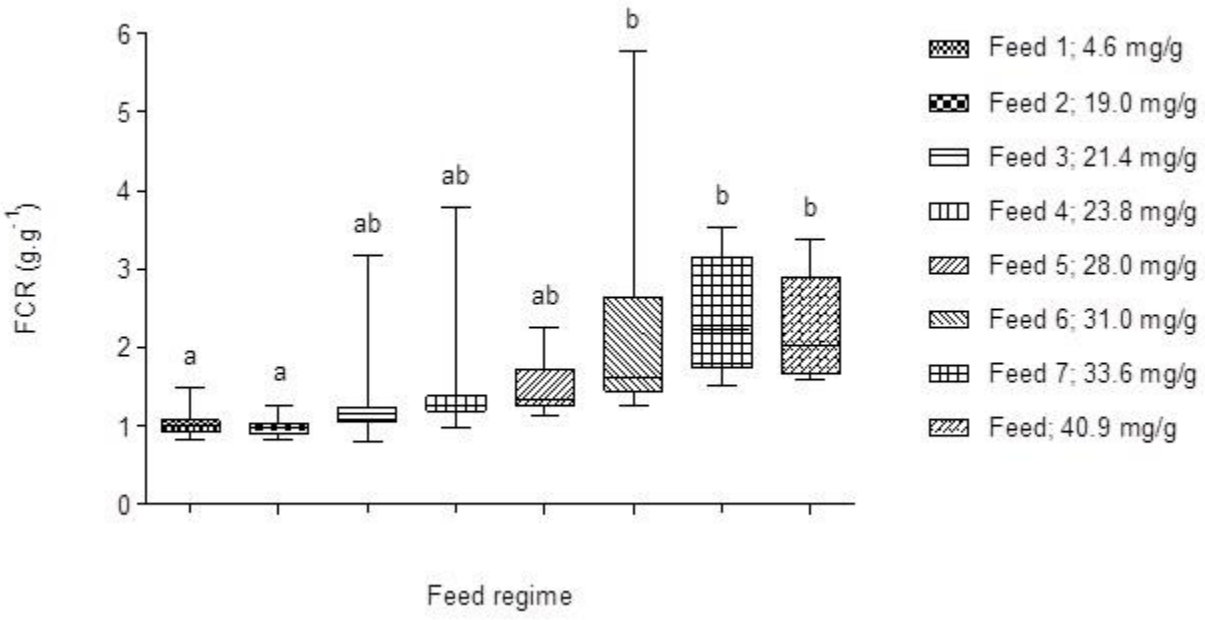

Figure 3.6 Feed conversion ratio (FCR) (n= 27) over the 50 day experiment for juvenile barramundi fed to satiation with one of eight experimental feeds containing supplementary TRP at inclusion rates displayed in the legend as $mg.g^{-1}$ dry weight. Values shown for each feed are minimum, 25^{th} percentile, median, 75^{th} percentile, and maximum. ANOVA showed difference between feed treatments ($F = 5.29$, *df* 7, 64, $p < 0.0001$). Differences in FCR between feed treatments are described by superscript letters (Tukey's multiple comparison test $p < 0.05$).

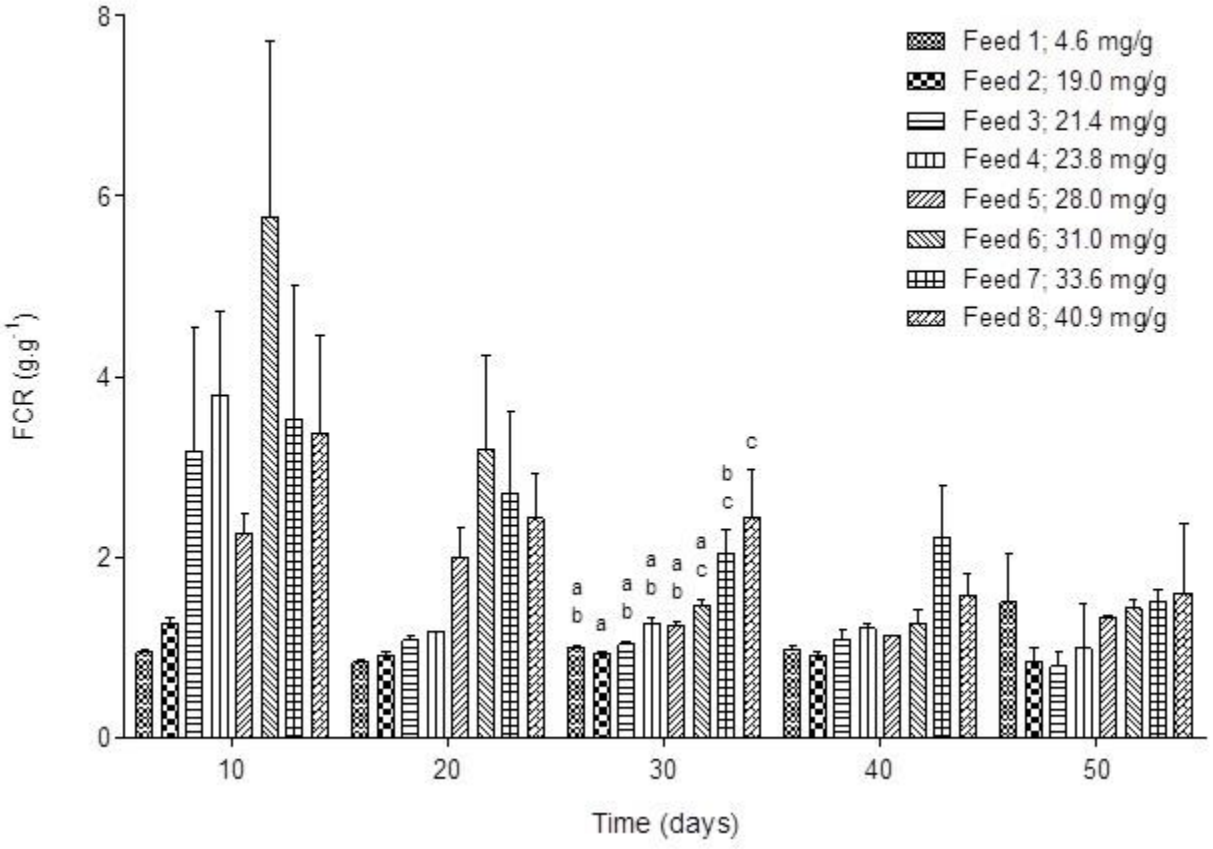

Figure 3.7 Mean feed conversion ratio (FCR) ± SEM (n=3) every 10th day calculated over the preceding 5 day period for juvenile barramundi fed to satiation with one of eight experimental feeds containing supplementary TRP at inclusion rates displayed in the legend as mg.g^{-1} dry weight. ANOVA showed significant difference between FCR's when compared to TRP content of the feed ($F = 6.04$, *df* 7, 16, $p < 0.01$). Differences in FCR between feed treatments were present only for day 30 data and are described by superscript letters (Tukey's multiple comparison test $p < 0.05$).

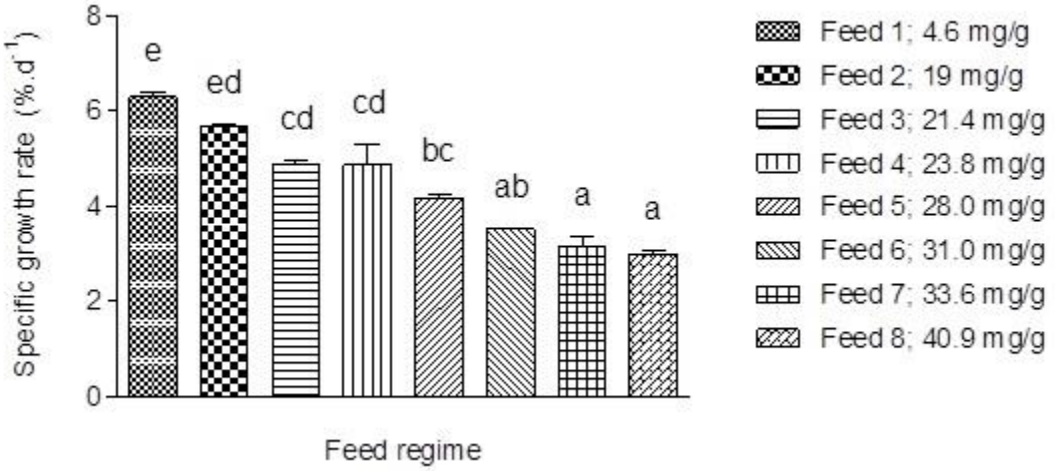

Figure 3.8 Specific growth rate (%.d^{-1}) of juvenile barramundi fed to satiation on one of eight experimental feeds containing supplementary TRP at inclusion rates displayed in the legend as mg.g^{-1} dry weight. Data are presented as mean ± SEM throughout a 50 day period. ANOVA showed a difference between treatments (F 42.8, *df* 7, 16, $p < 0.0001$). Different superscript letters denote differences between treatment means (Tukey's multiple comparison test $p < 0.05$).

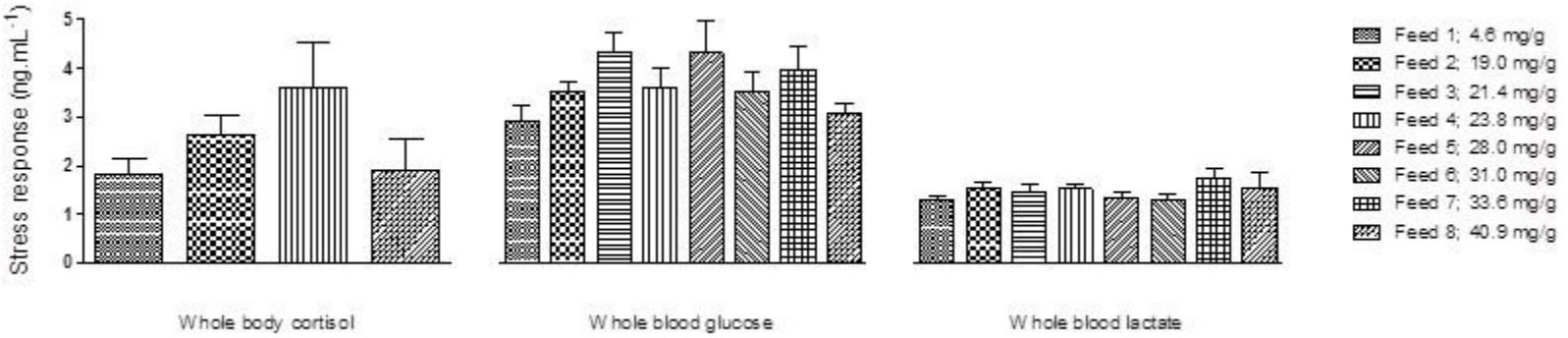

Figure 3.9 Physiological stress response at the conclusion of a 50 day experiment measured in whole body homogenate (cortisol) and whole blood (glucose and lactate) from juvenile barramundi fed to satiation with feeds containing supplementary TRP. Rates of TRP inclusion are displayed in the legend as $mg.g^{-1}$ dry weight. No differences ($p < 0.05$) were observed between treatments for cortisol, glucose or lactate when subjected to ANOVA.

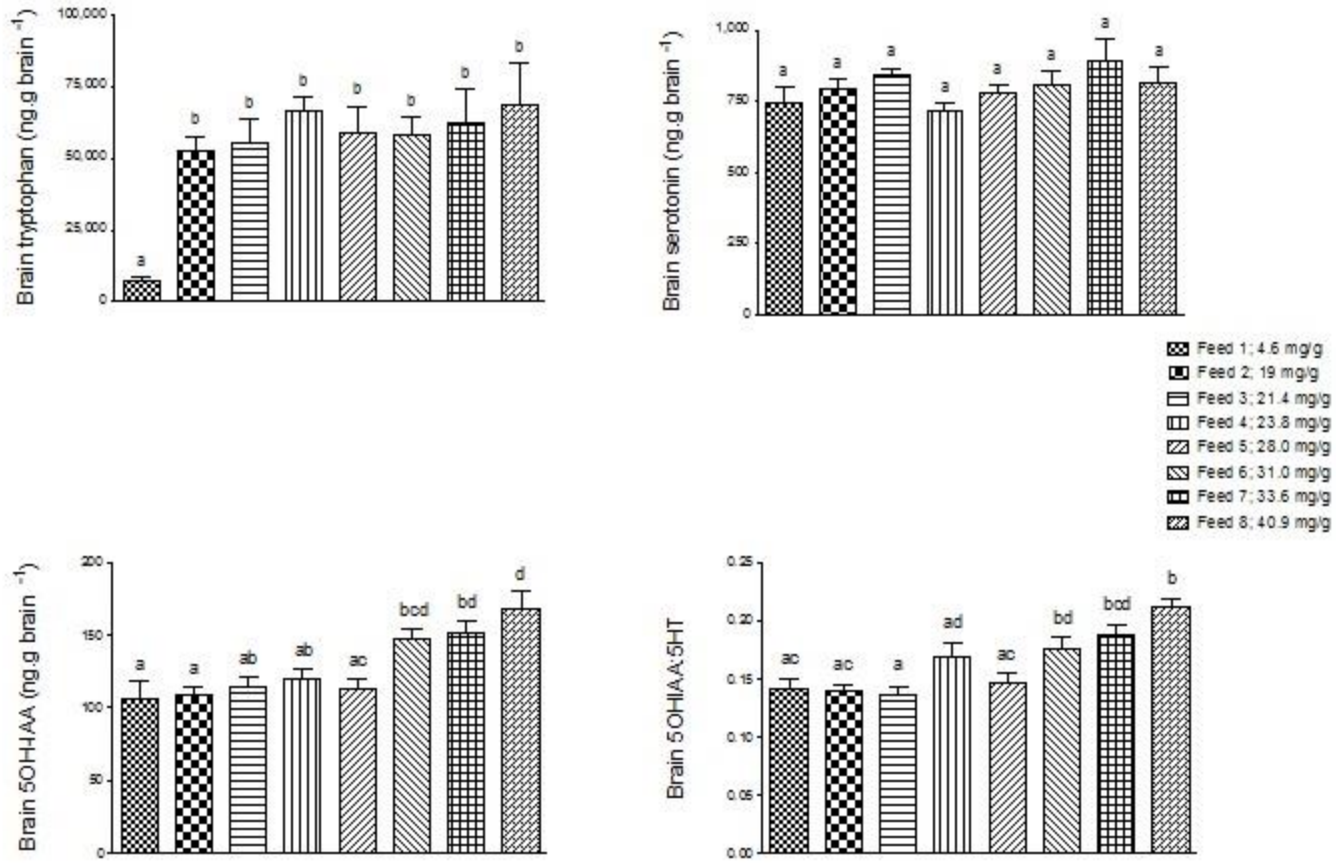

Figure 3.10 Tryptophan, associated neurotransmitters and metabolites extracted from whole brains of juvenile barramundi fed to satiation pelleted feed containing varying levels of supplementary TRP for 50 days. Data are presented as mean ± SEM. Rates of TRP inclusions are displayed in the legend as mg.g^{-1} dry weight. ANOVA showed a difference between feed treatments for brain TRP (F = 4.88, *df* 7, 110, p < 0.0001), brain 5OH-IAA (F = 7.67, *df* 7, 112, p < 0.0001), and the ratio of 5OH-IAA to 5HT (F = 9.45, *df* 7, 112, p < 0.0001). Differences in response between feeds are indicated by different superscript letters (Tukey's multiple comparison test p < 0.05).

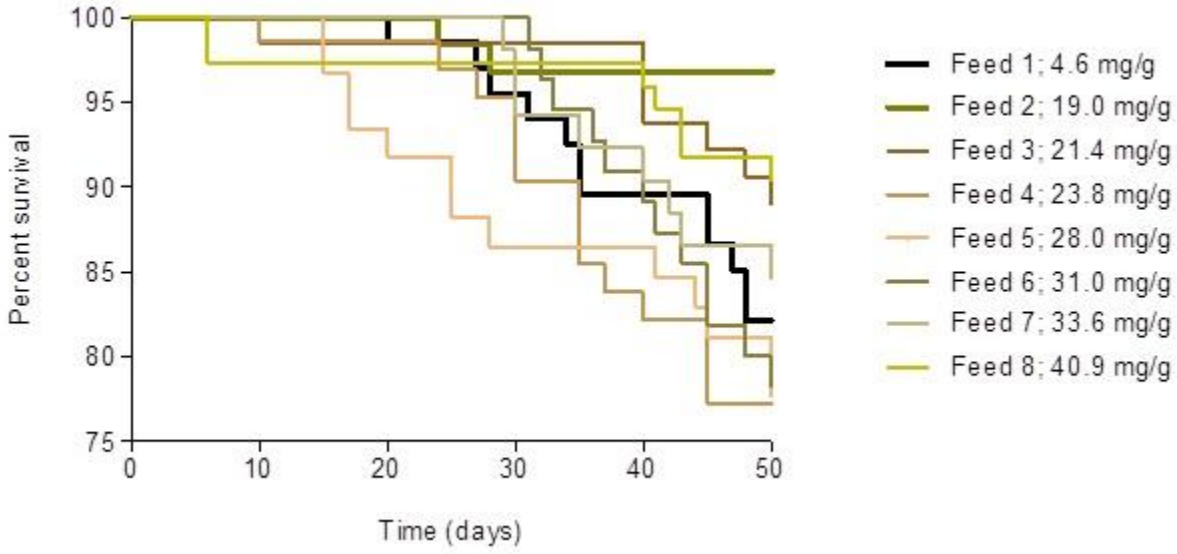

Figure 3.11 Cannibalism associated mortality presented as percent survival for juvenile barramundi fed to satiation with feeds containing supplementary TRP over 50 days. Data from each treatment are pooled across the three triplicate tanks and presented as a total. Censored data other than cannibalism associated mortality are included. No trend for response compared to dose was observed (Logrank test for trend). A difference between the curves (x^2 6.867, *df* 1, $p < 0.05$) was found to be between the reference feed (feed 1, 4.6 $mg.g^{-1}$) and feed 2 (19.0 $mg.g^{-1}$), ($p = 0.007$) (Log-rank Mantel-Cox test) after p value adjustment to 0.007 (Bonferroni) was made.

3.4 Results - Experiment 4

3.4.1 Food intake and growth performance

No differences were observed between treatments at the start of experiment 4 for either weight or length. See Table 3.4 (p 109). Both weight and length were affected negatively over time in a dose dependent manner when compared to increasing TRP content of the diet. Two-way ANOVA of both ration and dose showed a significant effect of both on final weight ($F = 1090$, *df* 1, 16, $p < 0.0001$; $F = 27$, *df* 3, 16, $p < 0.0001$), and on final length ($F = 1550$, *df* 1, 16, $p < 0.0001$; $F = 78$, *df* 3, 16, $p < 0.0001$) respectively. See Table 3.4 (p 109). Mean overall mortality across all feed rations and treatments, was 22.2 %. No mortality was observed other than cannibalism-associated mortality and no differences were observed in mortality between feed treatments within ration or between ration. See Table 3.4 (p 109) & Figure 3.21 (p 119).

Strong linear regressions were observed for (log transformed) wet weight (WW) and total length (TL) ($p <0.0001$) (Figure 3.12, A, p 110); body depth (BD) and total length ($p <0.0001$) (Figure 3.12, B, p 110); horizontal mouth gape (HG) and total length ($p <0.0001$) (Figure 3.12, C, p 110); vertical mouth gape (VG) and total length ($p <0.0001$) (Figure 3.12, D, p 110), horizontal mouth gape and body depth ($p <0.0001$) (Figure 3.12, E, p 110); and vertical mouth gape and body depth ($p <0.0001$) (Figure 3.12, F, p 110).

Food intake ($g.fish^{-1}.day^{-1}$) was intentionally affected by ration treatment. Two-way ANOVA of ration and dose found food intake to be negatively affected by TRP content of feed in a dose dependent manner ($F = 384$, *df* 7, 160, $p < 0.0001$) (Figure 3.13, p 111). When daily food intake was analysed relative to body weight no differences between feed treatments within ration

were observed over the 50 day experiment. See

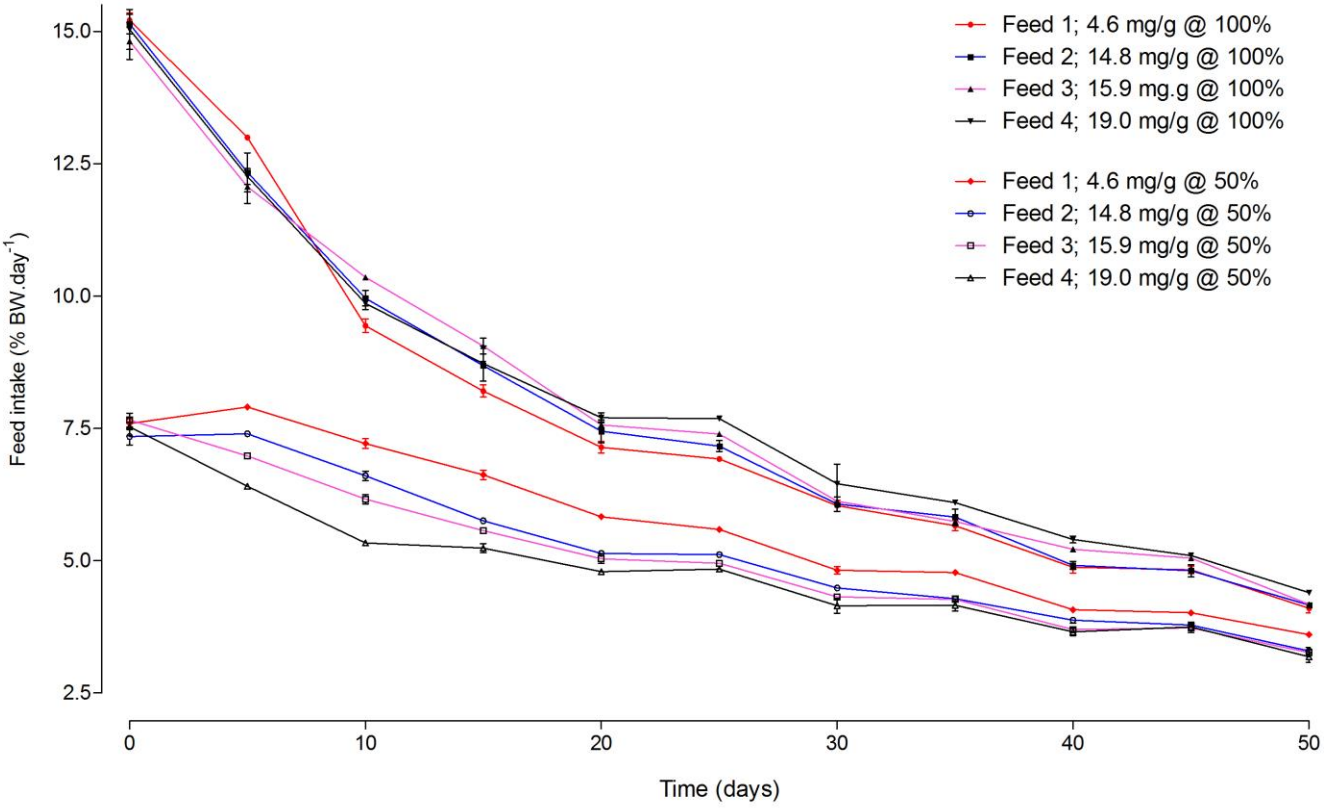

Figure 3.14 (p 112).

The daily ingestion of TRP reported as mg consumed per gram of wet weight of fish reduced over time for all treatments ($F = 3440$, *df* 10, 160, $p < 0.0001$) (Figure 3.15, p 113), in line with the expected and observed reduction in food intake as a percentage of BW over this size range. On day one extremely elevated values were recorded when compared to either the control or later values.

When FCR data calculated at 5 day intervals over the course of the experiment, starting at day 10 were subjected to ANOVA no differences were observed within feed treatment between ration. See Figure 3.16 (p 114Figure 3.8). Two way ANOVA of ration and feed treatment showed significant differences for feed treatment at day 10 ($F = 12.4$, *df* 3, 16, $p < 0.001$), day 15 ($F = 29.2$, *df* 3, 16, $p < 0.0001$), day 20 ($F = 4.83$, *df* 3, 16, $p < 0.05$), day 25 ($F = 7.63$, *df* 3, 16, $p < 0.01$), and a difference for ration at day 50 ($F = 5.66$, *df* 1, 16, $p < 0.05$) (Figure 3.17, p 115).

Specific growth rate (%.d^{-1}) was found to be strongly affected by ration, ($F = 720$, *df* 1, 16, $p < 0.0001$) and feed treatment with reductions observed with increasing TRP content of feed ($F = 720$, *df* 1, 16, $p < 0.0001$) (Figure 3.18, p 116).

3.4.2 Endocrine stress response

Ration and feed treatment did not affect cortisol, glucose or lactate (Two-way ANOVA) (Figure 3.19, p 117).

3.4.3 Neuroendocrine response

Ration ($F = 14.19$, *df* 1, 107, $p < 0.001$) and feed treatment ($F = 46.05$, *df* 3, 107, $p < 0.0001$) affected brain [TRP] (Two-way ANOVA, Figure 3.20, p 118). Ration ($F = 10.79$, *df* 1, 108, $p < 0.01$), and feed treatment ($F = 4.66$, *df* 3, 108, $p < 0.01$) affected brain [5-HT] (Two-way ANOVA, Figure 3.20, p 118). Ration ($F = 4.06$, *df* 1, 111, $p < 0.05$) and feed treatment ($F = 8.38$, *df* 3, 111, $p < 0.0001$) affected brain [5OH-IAA:5-HT] (Two-way ANOVA, Figure 3.20, p 118). Brain concentrations of 5OHIAA were found to be affected by feed treatment ($F = 9.76$, *df* 3, 108, $p < 0.0001$), but not by ration (Two-way ANOVA, Figure 3.20, p 118).

3.4.4 Survival

Mean survival of fish across all treatments was 78.8 % and neither ration size nor dose significantly affected survival. See Figure 3.21 (p 119).

Table 3.4 Performance of juvenile barramundi fed a ration of either 100% or 50% of previously observed satiation. Fish were fed one of four experimental feeds containing supplementary TRP at inclusion rates displayed in the legend as $mg.g^{-1}$ dry weight for 50 days. Differences between growth performance means are identified by differing letters (Tukey's multiple comparison test, $p < 0.05$).

		Weight (g)		Length (mm)		Survival (%)			
		Initial	Final	Initial	Final	Overall survival	Cannibalism		
Feed 1 (4.6 mg.g^{-1} TRP) @ 100%		0.263 ± 0.0043 $^{N=75}$	12.2 $^{(f)}$ ± 0.351 $^{N=62}$	27.2 ± 0.102 $^{N=75}$	96.6 $^{(f)}$ ± 0.949 $^{N=62}$	82.6	17.4	17.4	
Feed 2 (14.8 mg.g^{-1} TRP) @ 100%		0.264 ± 0.0033 $^{N=75}$	11.6 $^{(f)}$ ± 0.335 $^{N=51}$	27.6 ± 0.112 $^{N=75}$	93.6 $^{(ef)}$ ± 0.932 $^{N=51}$	68.0	32	32	
Feed 3 (15.9 mg.g^{-1} TRP) @ 100%		0.261 ± 0.0033 $^{N=75}$	10.5 $^{(e)}$ ± 0.245 $^{N=57}$	27.3 ± 0.113 $^{N=75}$	89.9 $^{(e)}$ ± 0.756 $^{N=57}$	76.0	24	24	
Feed 4 (19.0 mg.g^{-1} TRP) @ 100%		0.257 ± 0.0050 $^{N=75}$	9.44 $^{(d)}$ ± 0.304 $^{N=62}$	27.2 ± 0.113 $^{N=75}$	85.5 $^{(d)}$ ± 1.04 $^{N=62}$	82.6	17.4	17.4	
Feed 1 (4.6 mg.g^{-1} TRP) @ 50%		0.264 ± 0.0036 $^{N=75}$	5.23 $^{(c)}$ ± 0.149 $^{N=59}$	27.4 ± 0.116 $^{N=75}$	74.8 $^{(c)}$ ± 0.846 $^{N=59}$	78.6	21.4		21.4
Feed 2 (14.8 mg.g^{-1} TRP) @ 50%		0.273 ± 0.0038 $^{N=75}$	3.88 $^{(b)}$ ± 0.109 $^{N=64}$	27.6 ± 0.117 $^{N=75}$	66.1 $^{(b)}$ ± 0.738 $^{N=64}$	85.3	14.7		14.7
Feed 3 (15.9 mg.g^{-1} TRP) @ 50%		0.261 ± 0.0039 $^{N=75}$	3.21 $^{(bc)}$ ± 0.105 $^{N=60}$	27.4 ± 0.117 $^{N=75}$	61.9 $^{(b)}$ ± 0.738 $^{N=60}$	80.0	20		20
Feed 4 (19.0 mg.g^{-1} TRP) @ 50%		0.266 ± 0.0034 $^{N=75}$	2.71 $^{(a)}$ ± 0.087 $^{N=58}$	27.6 ± 0.111 $^{N=75}$	58.2 $^{(a)}$ ± 0.724 $^{N=58}$	77.3	22.7		22.7
Total	F	-	-	-	-	x^2 10.24	x^2 10.24	x^2 6.63	x^2 1.98
	df	-	-	-	-	7	7	3	3
	p	-	-	-	-	0.176	0.176	0.085	0.577
Ration	F	1.96	1090	4.22	1550	- -	-	-	-
	df	1, 16	1, 16	1, 16	1, 16	- -	-	-	-
	p	0.181	< 0.0001	0.056	< 0.0001	- -	-	-	-
Dose	F	1.09	27	2.74	78	- -	-	-	-
	df	3, 16	3, 16	3, 16	3, 16	- -	-	-	-
	p	0.383	< 0.0001	0.078	< 0.0001	- -	-	-	-

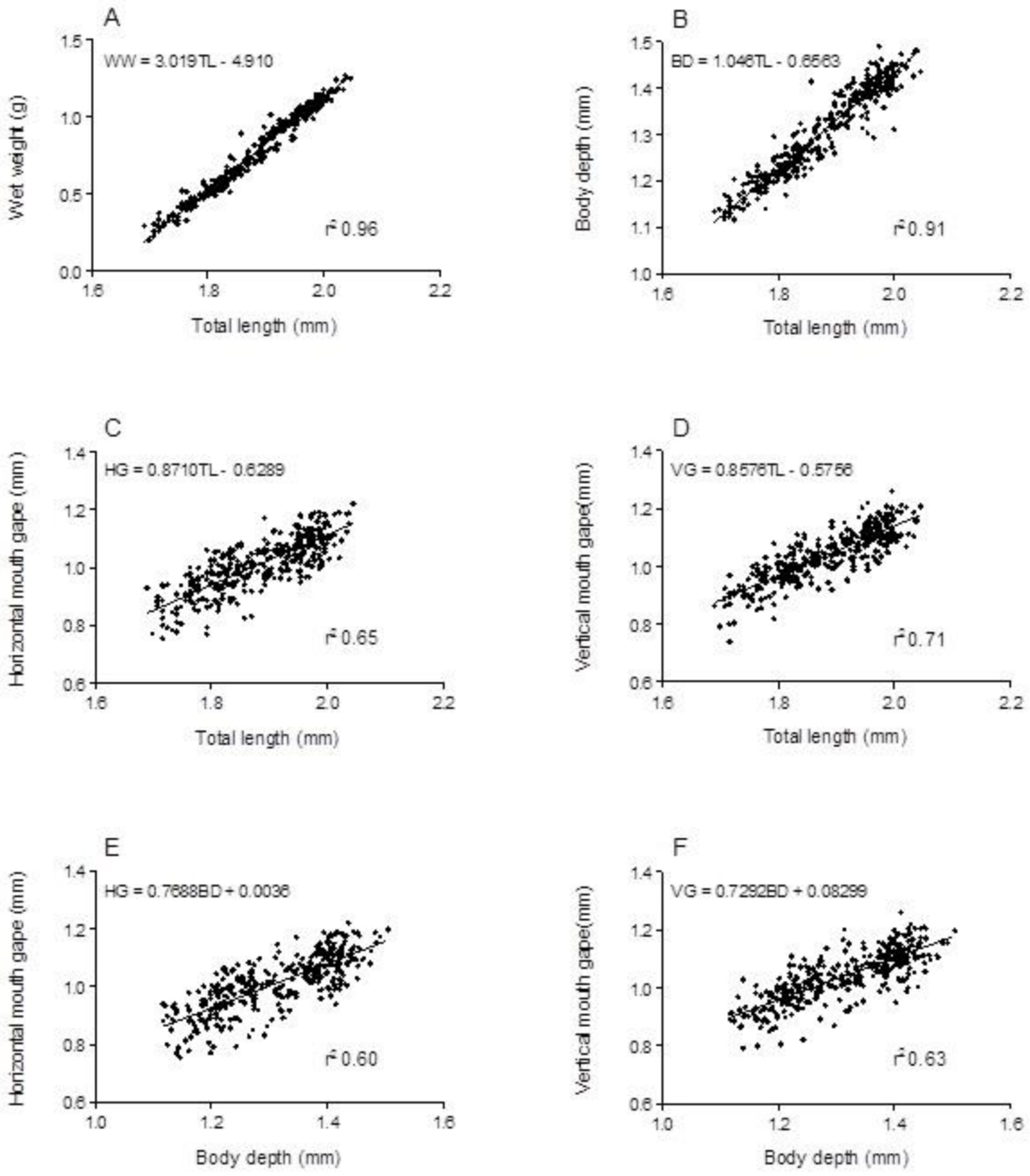

Figure 3.12 Linear regressions of log transformed size measurements of juvenile barramundi. A, wet weight (WW) and total length (TL) ($p < 0.0001$); B, body depth (BD) and total length ($p < 0.0001$); C, horizontal mouth gape (HG) and total length ($p < 0.0001$); D, vertical mouth gape (VG) and total length ($p < 0.0001$); E, horizontal mouth gape and body depth ($p < 0.0001$); and F, vertical mouth gape and body depth ($p < 0.0001$) in juvenile barramundi.

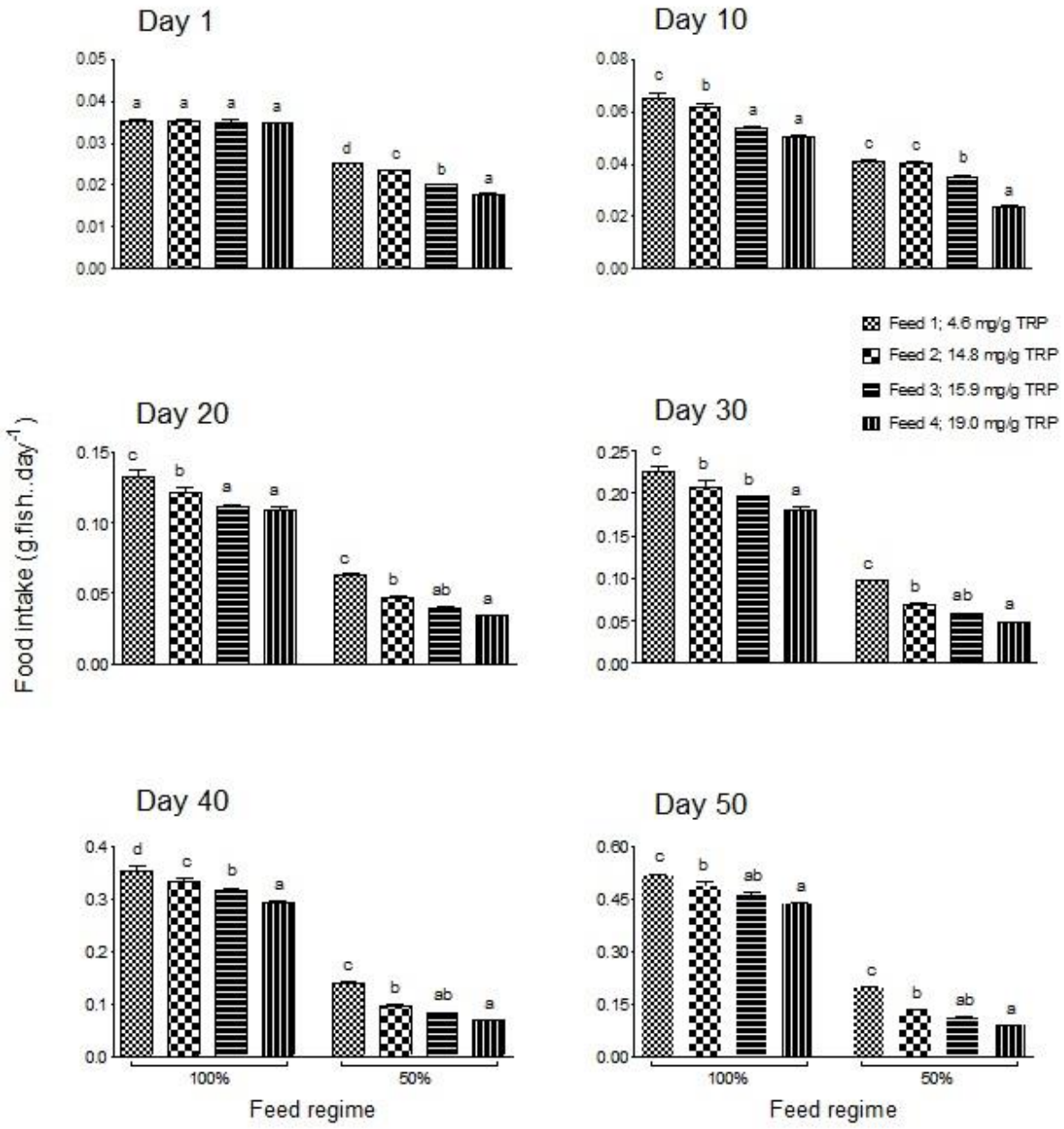

Figure 3.13 Mean daily food intake ± SEM (n=3) of juvenile barramundi fed a ration of either 100% (of feed 4, 19.0 mg.g^{-1}) or 50% of previously observed satiation food intake from experiment 3. Fish were fed one of four experimental feeds containing supplementary TRP at inclusion rates displayed in the legend as mg.g^{-1} dry weight. Data are presented as g.fish^{-1}.day^{-1} at 6 time points during the 50 day experiment. Two way ANOVA of ration and dose showed a significant effect on food intake of dose at day 1 ($F = 58.5$, *df* 3, 16, $p < 0.0001$), day 10 ($F = 97.4$, *df* 3, 16, $p < 0.0001$), day 20 ($F = 51.7$, *df* 3, 16, $p < 0.0001$), day 30 ($F = 65.6$, *df* 3, 16, $p < 0.0001$), day 40 ($F = 61.3$, *df* 3, 16, $p < 0.0001$), and day 50 ($F = 54.6$, *df* 3, 16, $p < 0.0001$). Differences in food intake between treatments at each time point are displayed by differing superscript letters (Bonferroni post test $p < 0.05$).

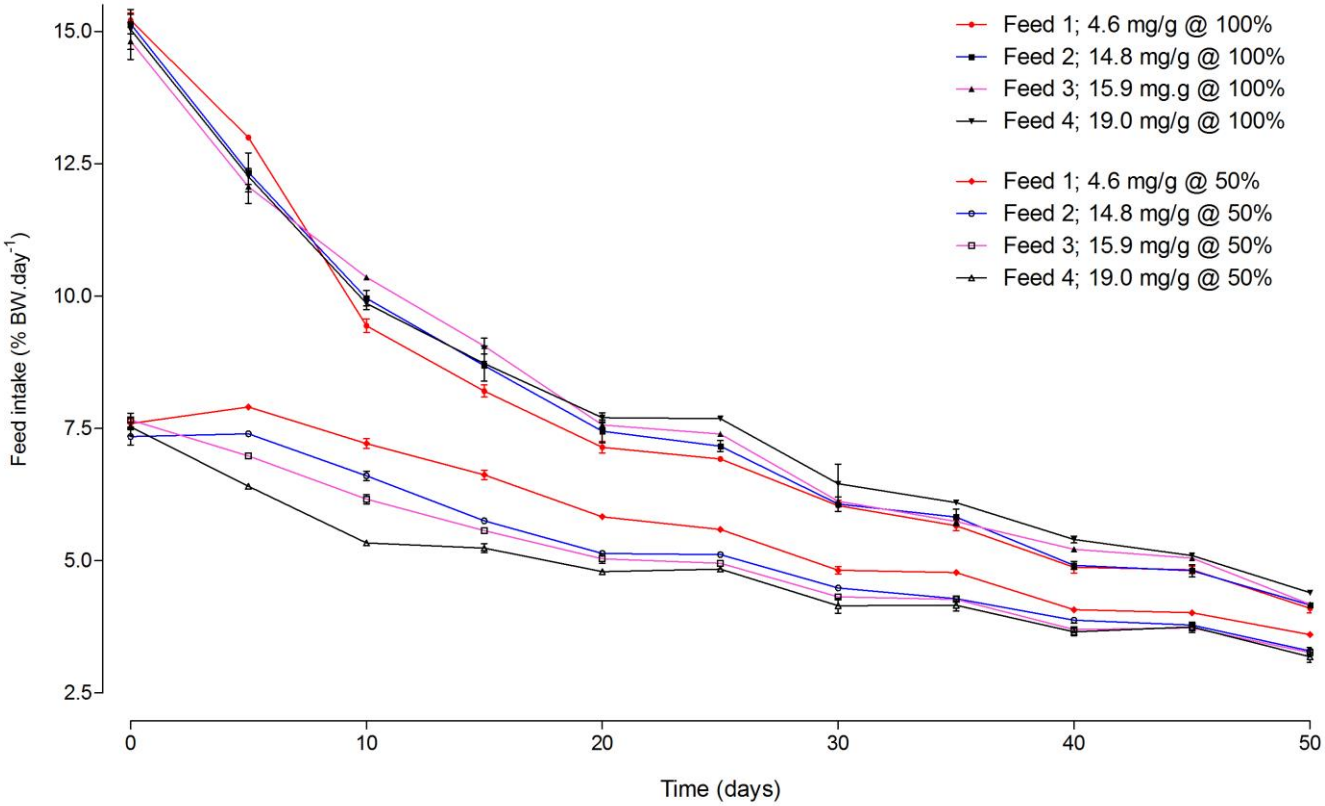

Figure 3.14 Mean daily food intake ± SEM of juvenile barramundi fed a ration of either 100% (of feed 4, 19.0 mg.g^{-1}) or 50% of observed satiation food intake from experiment 1. Fish were fed one of four experimental feeds containing supplementary TRP at inclusion rates of 1) 4.6; 2) 14.8; 3) 15.9, and 4) 19.0 mg.g^{-1} dry weight. Data are presented as percent of body weight (BW).day^{-1} calculated at 5 day intervals during the 50 day experiment.

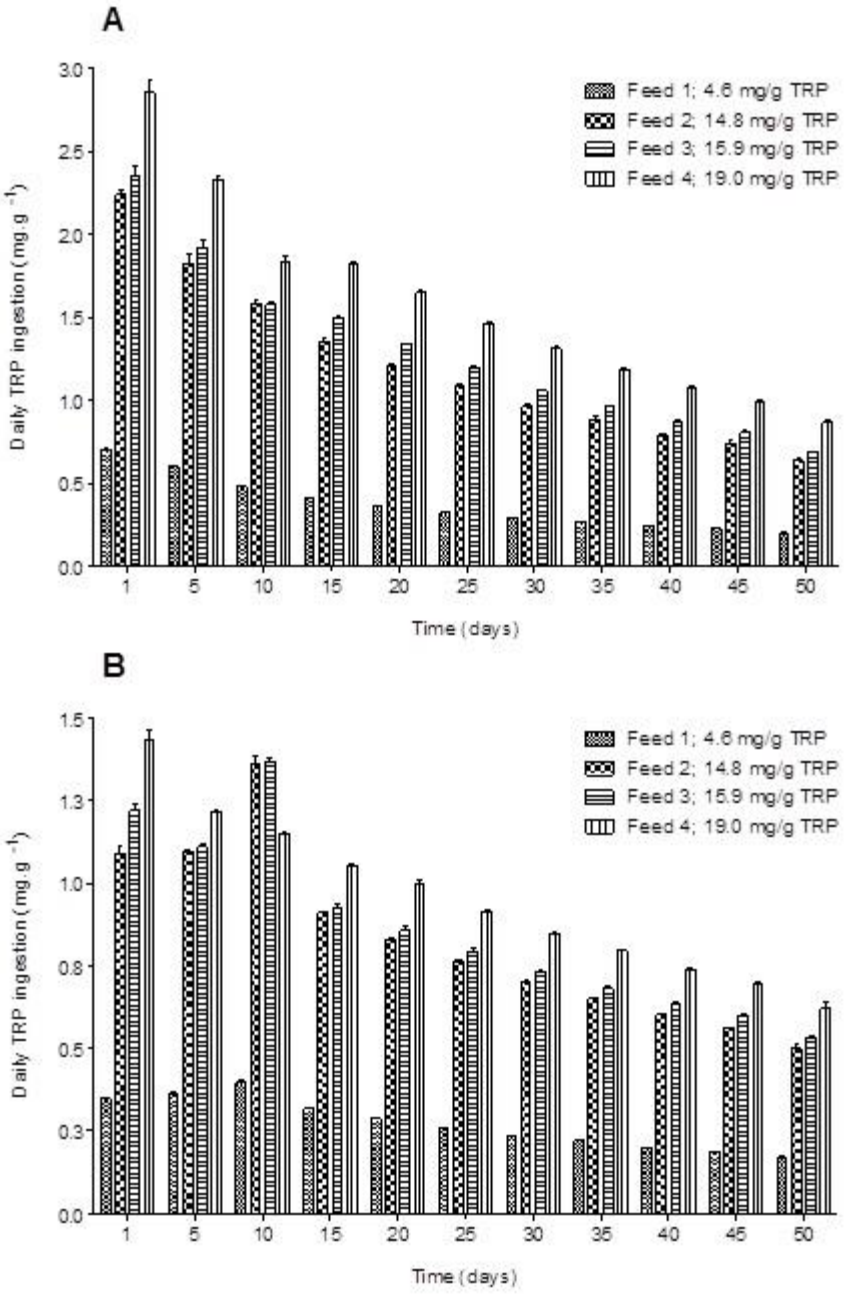

Figure 3.15 . Daily consumption of TRP per gram of wet weight of juvenile barramundi fed a ration of either (A) 100% (of feed 4, 19.0 mg.g^{-1}) or (B) 50% of observed satiation food intake from experiment 1 at inclusions shown in the legend at 11 time points during a 50 day experiment. Data are derived from daily tank food intake and average weights each five days, and are presented as mean (n=3) ± SEM.

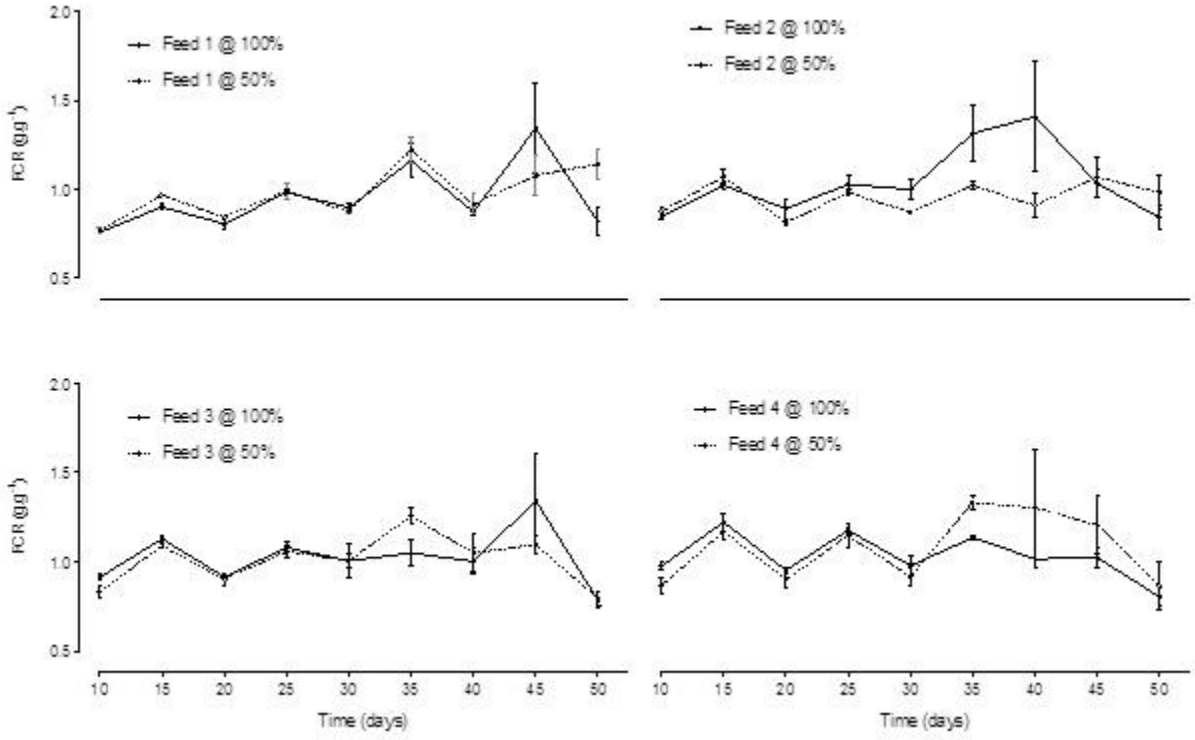

Figure 3.16 Mean feed conversion ratio (FCR) ± SEM for juvenile barramundi fed a ration of either 100% (of feed 4, 19.0 mg.g^{-1}) or 50% of observed satiation food intake from experiment 1. Fish were fed one of four experimental feeds containing supplementary TRP at inclusion rates of 1) 4.6; 2) 14.8; 3) 15.9, and 4) 19.0 mg.g^{-1} dry weight. Data are presented as weight of food consumed by all fish in each tank divided by weight gained, calculated at 5 day intervals during the 50 day experiment. No differences in FCR between ration were observed.

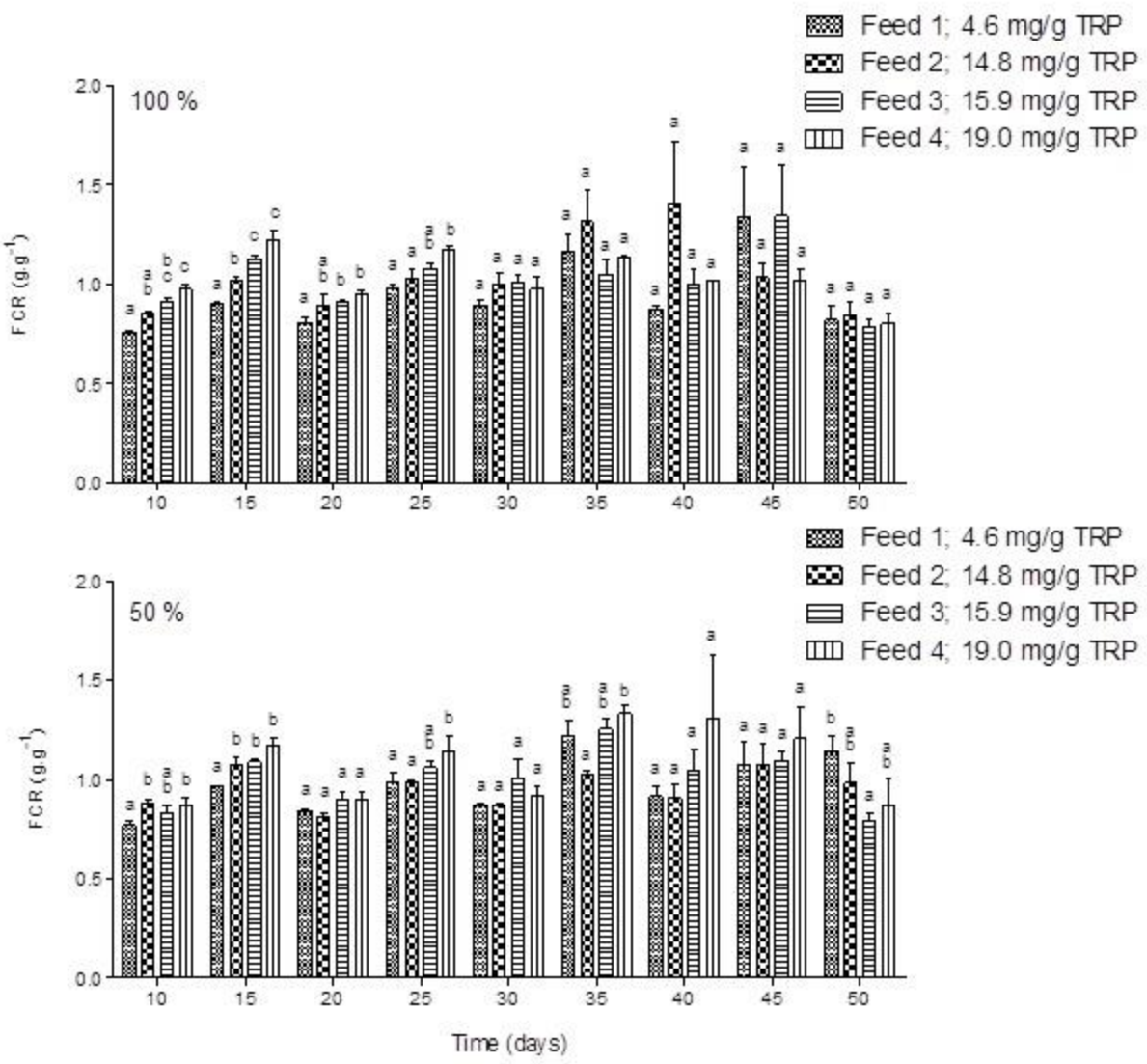

Figure 3.17 Mean feed conversion ratio (FCR) ± SEM starting day 10 calculated over the preceding 5 day periods for juvenile barramundi fed a ration of either 100% (of feed 4, 19.0 $mg.g^{-1}$) or 50% of observed satiation food intake from experiment 1. Fish were fed one of four experimental feeds containing supplementary TRP at inclusion rates displayed in the legend as $mg.g^{-1}$ dry weight. Two-way ANOVA of ration and feed treatment showed significant differences for feed treatment at day 10 ($F = 12.4$, *df* 3, 16, $p < 0.001$), day 15 ($F = 29.2$, *df* 3, 16, $p < 0.0001$), day 20 ($F = 4.83$, *df* 3, 16, $p < 0.05$), day 25 ($F = 7.63$, *df* 3, 16, $p < 0.01$), and a difference for ration at day 50 ($F = 5.66$, *df* 1, 16, $p < 0.05$). Differences at each timepoint in FCR between feed treatments within ration treatment are described by superscript letters (Bonferroni post test $p < 0.05$).

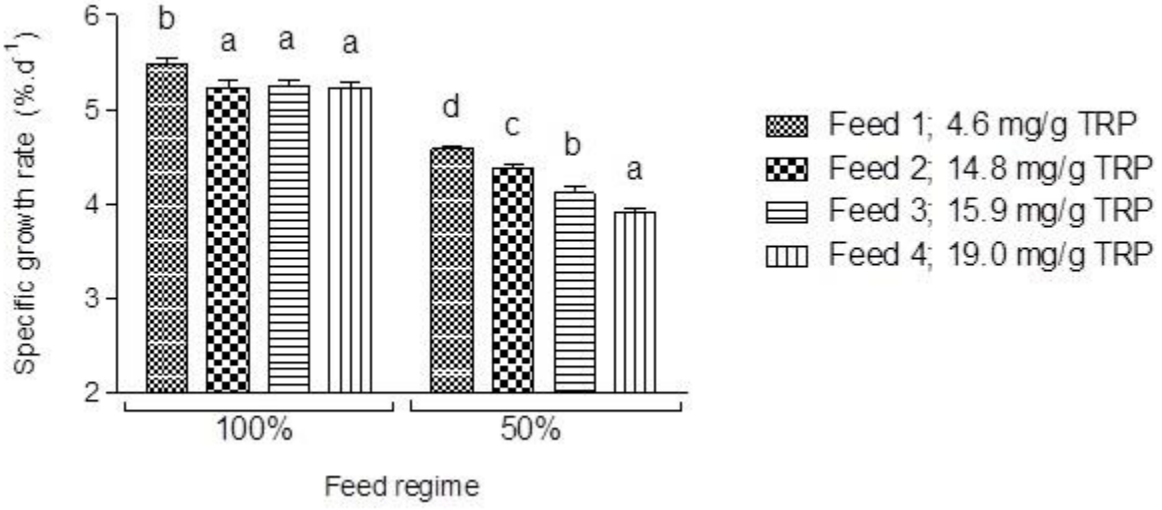

Figure 3.18 Specific growth rate (%.d^{-1}) of juvenile barramundi fed a ration of either 100% (of feed 4, 19.0 mg.g^{-1}) or 50% of observed satiation food intake from experiment 1. Fish were fed one of four experimental feeds containing supplementary TRP at inclusion rates displayed in the legend as mg.g^{-1} dry weight. Data are presented as mean ± SEM throughout a 50 day period. Two-way ANOVA of ration and feed treatment showed significant differences for both feed treatment ($F = 25.8$, *df* 3, 16, $p < 0.0001$) and ration ($F = 720$, *df* 1, 16, $p < 0.0001$). Different superscript letters denote differences between feed treatment means within ration (Bonferroni post test $p < 0.05$).

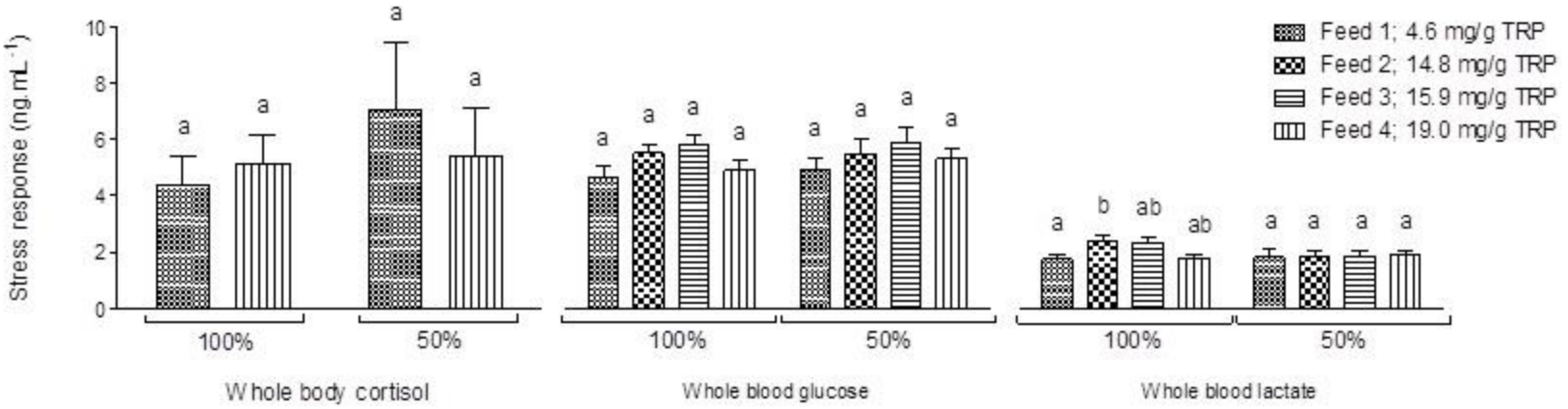

Figure 3.19 Physiological stress response presented as at the conclusion of a 50 day experiment measured in whole body homogenate (cortisol) and whole blood (glucose and lactate) from juvenile barramundi fed a ration of either 100% (of feed 4, 19.0 $mg.g^{-1}$) or 50% of observed satiation food intake from experiment 1. Fish were fed one of four experimental feeds containing supplementary TRP at inclusion rates displayed in the legend as $mg.g^{-1}$ dry weight. Data are presented as mean ± SEM. Two way ANOVA of ration and feed treatment showed no differences for either cortisol or glucose. Blood lactate concentrations were different between Feed 1 and Feed 2 for fish fed the 100% ration. Differences in physiological stress response between feed treatments within ration treatment are described by superscript letters (Bonferroni post test $p < 0.05$).

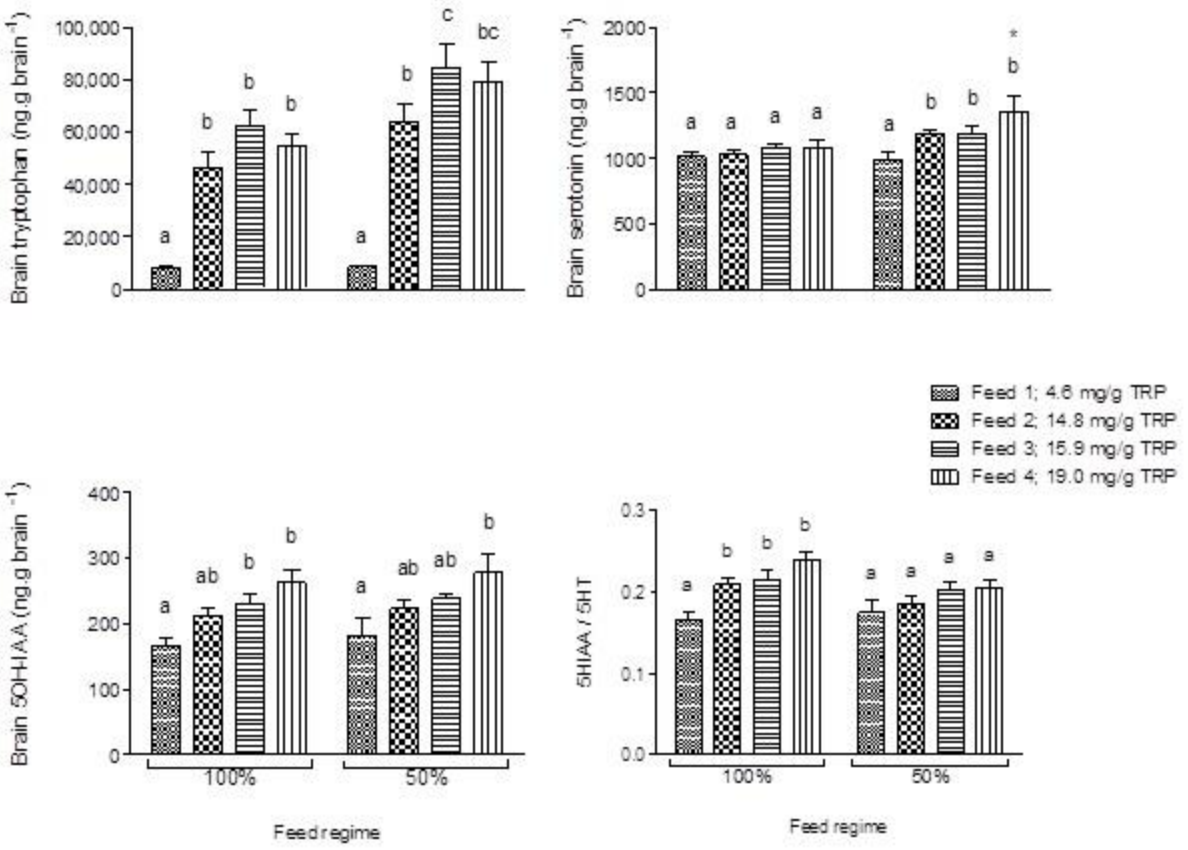

Figure 3.20 Tryptophan and associated neurotransmitters extracted from whole brains of juvenile barramundi fed a ration of either 100% (of feed 4, 19.0 mg.g^{-1}) or 50% of observed satiation food intake from experiment 1. Fish were fed one of four experimental feeds containing supplementary TRP at inclusion rates displayed in the legend as mg.g^{-1} dry weight for 50 days. Data are presented as mean ± SEM. Two way ANOVA showed ration ($F = 14.19$, *df* 1, 107, $p < 0.001$) and feed treatment ($F = 46.05$, *df* 3, 107, $p < 0.0001$) affected brain concentrations of TRP, in addition to 5HT: ration ($F = 10.79$, *df* 1, 108, $p < 0.01$), and feed treatment ($F = 4.66$, *df* 3, 108, $p < 0.01$), and 5OHIAA:5HT: ration ($F = 4.06$, *df* 1, 111, $p < 0.05$) and feed treatment ($F = 8.38$, *df* 3, 111, $p < 0.0001$). Brain concentrations of 5OHIAA were found to be affected by feed treatment ($F = 9.76$, *df* 3, 108, $p < 0.0001$), but not by ration. Differences in response between feeds are indicated by different superscript letters. Differences in response within TRP treatment but between ration treatment are indicated by an asterisk (*) (Tukey's multiple comparison test $p < 0.05$).

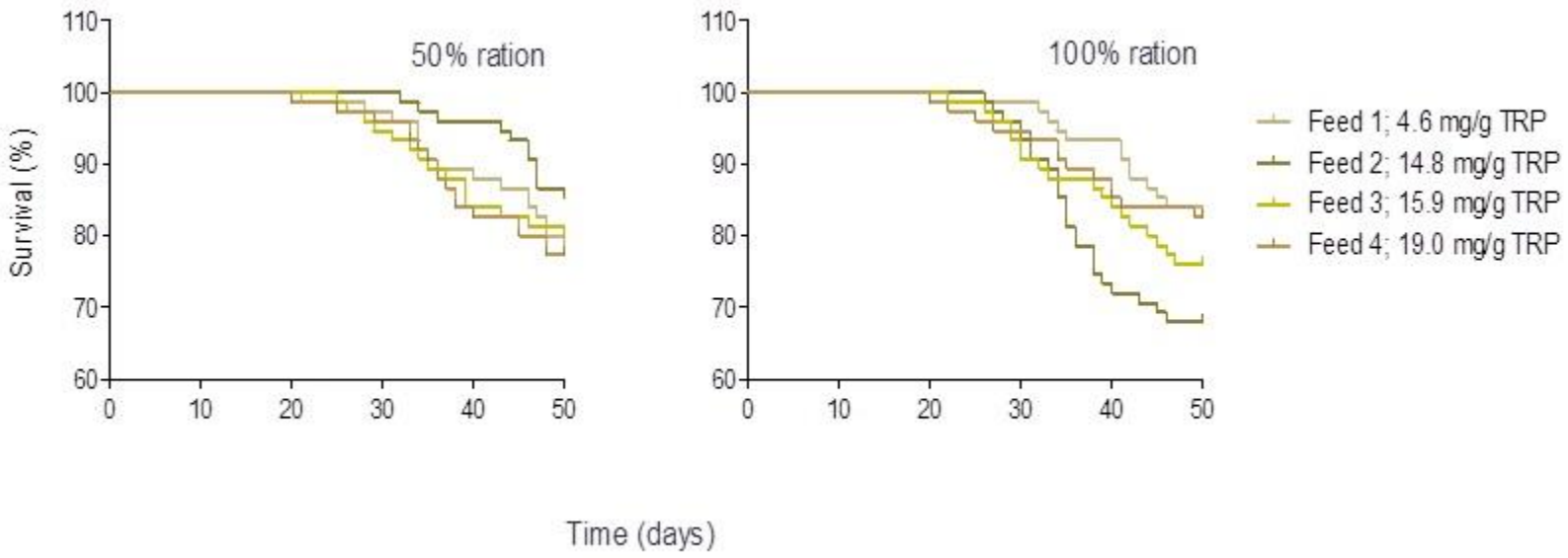

Figure 3.21 Cannibalism associated mortality presented as percent survival for juvenile barramundi fed a ration of either 100% (of feed 4, 19.0 mg.g^{-1}) or 50% of observed satiation food intake from experiment 1. Fish were fed one of four experimental feeds containing supplementary TRP at inclusion rates displayed in the legend as mg.g^{-1} dry weight for 50 days. Data from each feed treatment are pooled across the three triplicate tanks and presented as a total. No differences in cannibalistic mortality were observed between feed treatment or ration (Log-rank Mantel-Cox Test).

3.5 Discussion

The two experiments in the current study examined the effect of dietary TRP at levels ranging from 4.6 mg.g-1 to 40.9 mg.g-1 across two rations; satiation and half of satiation. In experiment 3 a satiation ration was delivered across a broad range of TRP inclusion and all TRP supplemented feeds were found to strongly inhibit food intake in a dose dependent manner. The onset of hypophagia was found to relate to TRP inclusion up to 28.0 mg.g-1. Above this level no differences were observed for the onset of hypophagia however differences were present in its intensity. Ingestion by fish of TRP measured in $mg.g^{-1}$ of body weight appeared similar between all supplemented feeds, especially between day 5 and day 20 suggesting the possibility of an upper tolerance level. This type of measure ($mg.g^{-1}$ body weight) of food intake does become unreliable as size differences between treatments become apparent. No differences in brain [TRP] were observed between TRP supplemented feed treatments, though a weak trend toward a positive correlation between the two could be suggested. However all TRP supplemented feed treatments produced substantially higher brain [TRP] than the concentrations observed for the reference feed. Despite reduced brain [TRP] in fish fed the reference feed compared to those fed the TRP supplemented feeds, no differences between feeds were present for brain [5-HT] suggesting a possible maximum capacity at evolutionary ambient TRP inclusion. Brain [5OH-IAA] showed differences between feed treatments and an apparent trend towards an increase concordant with dietary TRP inclusion, as did brain [5OH-IAA:5-HT] suggesting a faster turnover. Aggressive behaviour, measured by the rate and total level of cannibalism associated mortality did not appear affected by supplementing feed with TRP.

Experiment 4 focused on a narrow band of supplementary TRP inclusion at the lowest and lower levels of those studied in experiment 3. Differences between ration sizes were examined by delivering a satiation and a 50% ration. Measures of food intake and consequential levels of TRP ingestion reflected feeding regimes. Specific growth rate (SGR) was better for fish fed the reference feed at both rations than it was for fish fed TRP supplemented feeds, and at the 50% ration SGR reflected dose, however no differences between SGR for TRP supplemented feeds

were evident for the satiation ration. This confirms a trend for a poorer feed conversion ratio with increased TRP supplementation. A substantial increase in brain [TRP] was evident in fish fed supplemented feed when compared to fish fed the reference feed. No differences in brain [TRP] between levels of supplementation were present for the satiation ration, however they were at the reduced ration. Brain [5-HT] was slightly higher in experiment 4 than in experiment 3, without difference between dose for the satiation ration, but exhibited a trend toward increased concentration as feed supplementation increased at the 50% ration. These highlighted points will be discussed in more detail below.

3.5.1 Food intake and growth performance

Barramundi exhibit very high food intake at early stages of development (up to 5 g) decreasing quite quickly with time. The percentage of food consumed per day relative to body weight decreases as fish grow larger and their metabolic demands decrease (Goddard 1996). The satiation ration offered to fish fed the reference feed (feed 1, 4.6 $mg.g^{-1}$ TRP) in experiment 3 ranged from 15.29% $BW.day^{-1}$ at the start of the experiment to 4.89% $BW.day^{-1}$ at the culmination. In light of this, food intake is presented as both grams consumed per fish per day and as percent of body weight consumed per fish per day to provide where possible the most appropriate measure. Furthermore, food intake of TRP supplemented feed is at times discussed in reference to observed consumption of the reference feed at the relevant size.

The lack of difference in food intake between treatments at both mealtimes on day 1 indicates the hypophagic effect of supplementary dietary TRP seen later in the experiment is not as a result of poor palatability, nor is it significantly mediated within an eleven hour range. From the morning mealtime on day 5 food intake of fish fed feed 1 (4.6 $mg.g^{-1}$ TRP) was greater than for all feeds, including feed 2 (19.0 $mg.g^{-1}$ TRP), and remained higher for the duration of the experiment. This indicates that that the time response of TRP mediated hypophagia is dose-dependent and commences for feeds containing 28.0 $mg.g^{-1}$ or more of TRP between 11 and 24 h after initial exposure. Food intake measured as grams per fish per day is strongly associated

with the size of the fish, with larger fish capable of consuming much greater amounts than smaller fish and thus, in isolation measurements of food intake in this way must be treated with caution when significant differences in fish size between treatment are present. In the current situation it is appropriate as, early in the experiment, differences in fish size between feed treatments are yet to emerge.

As explained previously, barramundi exhibit very high food intake as a percentage of body weight whilst small. This rate of food intake reduces rapidly as the fish grow. Those fish consuming higher TRP feeds did not grow, so the percent of food consumption did not change. The only significant differences between feed treatments were between the reference feed (Feed1, 4.6 $mg.g^{-1}$ TRP) and the two feeds with the highest TRP inclusion, feeds 7 (33.6 $mg.g^{-1}$ TRP) and 8 (40.9 $mg.g^{-1}$ TRP). It could easily be inferred from this that there were no differences in overall food intake of feeds 1 through 6 however this assumption could not be further from the truth.

Supplementary dietary TRP negatively impacted on food intake in a dose dependent manner as can be evidenced from the overall consumption of feed reducing in a linear fashion from 1.74 Kg for the reference feed to 91 g for feed 8. Linear regression of food intake (% $BW.day^{-1}$) on mean wet weight (g) provides a more accurate measure of food intake relative to TRP content of the feed. It shows that the differences between the y intercepts are extremely significant.

The association between up-regulation of the serotonergic system and depressed food intake has been documented in a number of species including rats, chickens and humans and has been reviewed (Blundell and Halford 1998). In fish however experiments studying food intake relative to TRP content are rare, and published results in the field are at best species-specific and at worst inconclusive. No differences in food intake of rainbow trout were observed when feeds were supplemented with TRP at up to 5.9 $mg.g^{-1}$ (a much lower inclusion that the current experiments) and delivered for up to 98 days (Johnston *et al.* 1990; Lepage *et al.* 2003). A higher supplementary inclusion of 15.0 $mg.g^{-1}$ TRP (within the range studied in experiment 2) also failed to have an impact on food intake in rainbow trout over 3 or 7 days (Winberg *et al.* 2001). Furthermore, feed TRP inclusion of 9.5 $g.Kg^{-1}$, 18.4 $g.Kg^{-}1$, and 35.7 $g.Kg^{-}1$ was also found

to have no impact on food intake by rainbow trout (Lepage *et al.* 2002). None of these experimental designs detail whether fish were fed to satiation. As the food intake both prior to and during TRP supplementation phases appears lower than might be expected for the species at the described size, long term sub-satiation delivery may have obscured a hypophagic effect at the higher doses. In another experiment on rainbow trout, fish were fed to satiation with feed containing 24.7 $mg.g^{-1}$ TRP over 77 days and food intake was found to increase, though growth (SGR) and efficiency (FCR) were reduced (Papoutsoglou *et al.* 2005). In the previous experiment daily food intake is not presented as a percentage of body weight however daily TRP ingestion per fish data suggest that overall food intake, as a percentage of body weight, was low. Coloso *et al.* (2004a) found that barramundi fed a restricted ration had reduced growth (SGR) and efficiency (FER) at very low (1.1 $mg.g^{-1}$) total TRP inclusion, presumably as TRP became limiting rather than as an effect of 5-HT induced hypophagia, but didn't test any feed with more than 6.1 $mg.g^{-1}$ TRP. A reduction in growth was also noted in orange spotted grouper (*Epinephelus coioides*) fed to satiation with feed supplemented at 2.5, 5.0 and 10.0 $mg.g^{-1}$ TRP over 10 days however no data analysis of food consumption was provided (Hseu *et al.* 2003). Apparently a negative effect of TRP supplementation at 5, 7.5 and 10 $mg.g^{-1}$ on growth in mud crabs (*Scylla serrata*) was evident however data appear contradictory (Leopoldo *et al.* 2010). An increase in food intake post-stressor was observed in brown trout (*Salmo trutta*) fed TRP supplemented feed at 3.0 $mg.g^{-1}$ compared to a non-TRP supplemented feed, however food intake was quantified by feeding response, (Hoglund *et al.* 2007) rather than intake, and observed food intake was extremely low compared to expected levels for fish of this size.

In the current experiments reduced food intake was the main driver in decreasing growth performance relative to TRP content of the feed. The efficiency of food utilisation measured as FCR was also found to be dose-dependent relative to TRP content of the feed with more favourable, lower FCR values associated with lower TRP inclusion. It is acknowledged that FCR and dietary TRP content are closely related and may be co-linear and confounded but that is impossible to tease apart with the available data. FCR recorded at ten day intervals during experiment 3 showed a clear trend toward poorer conversion in feeds with higher TRP inclusion

confirmed by a strong correlation between increased inclusion and increased FCR, ($p < 0.0001$). At the lower TRP inclusion rates examined in experiment 4 no differences in FCR between feeds were observed when assessed across the duration of the study either between feeds or between rations. This response is in agreement with a study on similar sized barramundi fed one of six rations from starvation to satiation (Glencross 2008). Glencross (2008) observed no differences between FCR's for the larger 4 rations, with poorer FCR's only visible for the lowest feed delivery and the starvation ration. Notably the best (lowest) FCR was not from the satiation ration. In the current studies apparent digestibility of the feeds at different rations was not examined, however it is possible that some of the total digestible energy at high feed intake was wasted, and FCR may have been improved for feed 1 in experiment 3 at a slightly lower daily intake. When examined at 5 day intervals however the trend for reduced FCR was clear with significant differences in FCR observed between feeds at many timepoints especially over the first 25 days. A weak correlation was observed between FCR and TRP inclusion for 100% ration feeds though no such correlation was observed for fish fed a 50% ration. Reduced FCR for fish fed feed with supplementary TRP in experiment 4 was reflected in reduced food intake as food intake was calculated as a percentage of body weight and growth was depressed for fish consuming TRP supplemented feeds compared to those offered the reference feed.

At the relatively low inclusions and restricted rations of experiment 4 the amount of TRP (mg) ingested each day reduced over time when compared to the weight of the fish (g) reflecting the reducing daily food intake as a percentage of body weight. This would be reflected in a proportionate reduction in circulating TRP available to cross the blood brain barrier (BBB) which may lead to a corresponding reduction in serotonergic activity. Conversely, the timecourse of feeding inhibition suggests elevated circulating TRP may enhance neuron firing rate and / or receptor activity, thus requiring less TRP to deliver an equal or increased level of activity. Lepage *et al.* (2003) evaluated the time course of effects of dietary TRP on plasma cortisol levels in rainbow trout and expected the effect of elevated dietary TRP intake on 5HT synthesis and release to be very rapid; the up-regulation of the 5HT system has been observed in rainbow trout after exposure to only 15 s of handling stress (Gesto *et al.* 2013). However a reduction in post-stress plasma cortisol, determined to be as a result of TRP supplemented feed was evident

at day 7 but not days 3 or 28 (Lepage *et al.* 2003). The authors go on to suggest that long term dietary TRP may activate compensatory mechanisms, normalizing brain [TRP], and mistakenly state that after 28 days of TRP supplementation, [TRP] in both the telencephalon and the optic tectum were no different from that of fish fed control feed. Effects on aggression in rainbow trout fed TRP supplemented feed were similarly present at 7 days but not at 3 (Winberg *et al.* 2001). These authors also expect a rapid TRP induced elevation in serotonergic activity and propose that effects on 5-HT receptor mechanisms may be involved in the delayed effect. No effects on food intake were observed in either of the previous (Winberg *et al.* 2001; Lepage *et al.* 2003) studies however food intake and [TRP] were both low respectively. Lepage *et al.* (2003) claim to supplement with TRP at 8-fold natural inclusion however their total TRP is detailed at 3.57 $g.kg^{-1}$, a little less than natural inclusion. This may be a typo and should be read as 3.57 $g.100\ g^{-1}$ or it may explain the lack of observed effect of feed composition on brain serotonergic activity.

The exact mechanisms which trigger observed behavioural effects as a result of supplementary dietary TRP mediated serotonergic activity are not completely understood despite 3 decades of inquiry. The current experiment is the only one known to the author which details the time course of dietary TRP mediated hypophagia. If supplementary dietary TRP mediated serotonergic activity is proposed as a primary factor in hypophagia, reduced physiological stress response, and reduced aggression it is possible that the time course of up-regulation of the 5-HT system is reflected by the time to hypophagia observed in experiment 3, as previously described.

Contrary to normal expectations a trend for increased daily food intake as a percentage of body weight was observed for the higher dose feeds over time in experiment 3. This increase needs to be viewed within the context of severe hypophagia and might suggest that some acclimation to extreme TRP supplementation may have occurred over time. Though feeding continued, albeit severely reduced, intake appears to have been inhibited by TRP at a daily ingestion of around 2 $mg.gBW^{-1}$ for all supplemented feeds. This effect was particularly strong over the first

20 days of the experiment which is attributed to a more reduced variability in size between treatments at this stage, as well as possibly some acclimation to elevated circulating TRP.

3.5.2 Neuroendocrine response

TRP competes with other large neutral amino acids (LNAA) for passage across the BBB via a common transporter, for example in rainbow trout elevated plasma L-tyrosine reduced the uptake of L-tryptophan into the brain (Aldegunde *et al.* 1998), and thus the amount of food ingested can be a factor in brain [TRP]. Aldegunde *et al.* (1998) go on to show that uptake of intravenously delivered plasma TRP to the brain occurred at 30 minutes post-administration. Fish were fasted and TRP doses ranged from 12.5 to 200 $mg.kg^{-1}$. Brain [TRP] increased linearly however no differences were observed between the three upper doses, 100, 150 and 200 $mg.kg^{-1}$. In the current study (experiment 2) TRP was ingested by fish in the control group at between 700 $mg.kg^{-1}$ and 200 $mg.kg^{-1}$ at the 100% ration, and at between 350 $mg.kg^{-1}$ and 170 $mg.kg^{-1}$ at the 50% ration. Though the doses and delivery methods between studies are not directly comparable, it is possible that the lower doses delivered by Aldegunde *et al.* (1998) are within those expected as a result of normal food ingestion and the doses above 87 $mg.kg^{-1}$ (the last dose at which a linear response between dose and brain [TRP] was observed) exceed it. The current data suggest an upper limit for brain [TRP] in fish offered feed to satiation twice daily, possibly mediated by LNAA competition at the BBB. The trend for increasing brain [TRP] relative to increased supplementary inclusion for fish fed at a 50% restricted ration appears to confirm that supplementary TRP is obstructed from passage across the BBB.

No differences were observed in brain [5-HT] for fish fed to satiation twice daily, or the 100% ration, however a trend for increased brain [5-HT] compared to increased dietary inclusion, and differences between doses were observed at the 50% restricted ration. The lack of brain [5-HT] differences between the reference feeds and TRP supplemented feeds at both rations studied higher than 50% of satiation, despite very large differences in brain [TRP], might point to an upper limit or saturation point for 5-HT. This however seems unlikely as average brain [5-HT] at the 100% ration in experiment 4 was higher than those for satiation feeding in experiment 1, while those for the 50% restricted ration were higher still. This suggests other factors

associated with ration size, such as the relative concentrations of LNAA's in the brain, or saturation of the 5-HT neurons negatively impacts biosynthesis of 5-HT. Serotonin receptors are able to sense extracellular [5-HT] and respond by inhibiting both its synthesis and release (Best *et al.* 2010). It has been shown that if rats are given doses of TRP sufficient to raise brain [TRP] well beyond the normal range, the firing frequencies of the 5-HT releasing raphe neurons decreased markedly, however when given smaller doses of TRP sufficient to increase brain[TRP] within normal peaks no such reduction was observed (Wurtman 1988). At the satiation rations of the current experiments competition with other LNAA's for BBB transporter sites may have been affected despite the assumed greater proportion of blood TRP compared to other LNAA's. It seems unlikely that other LNAA's were affecting BBB transport of TRP to such a degree at the 50% ration, and the apparent trend for increased brain [5-HT] with increased brain [TRP], even at brain [TRP] greatly above normal, suggests LNAA competition may be the primary factor.

Differences in brain [5HIAA] were observed in a dose dependent manner relative to TRP content of the feed across both experiments and all rations irrespective of similarities observed for brain [5-HT]. Thus the rate of metabolism of 5-HT must be reasonably expected to be increased when compared to the TRP content of the ingested feed suggesting a faster rate of 5-HT turnover, despite a proposed reduction in 5-HT neuron firing frequency. Very similar [5HIAA] between feed ration treatments in experiment 4 further emphasise that elevated brain [TRP] increase serotonergic activity despite apparently low or similar [5-HT].

Serotonergic activity is most commonly described by the ratio of 5-HT to its primary metabolite 5HIAA as this indicates the rate of turnover(Cubitt *et al.* 2008). This ratio appears most affected by [5HIAA] as [5-HT] variability is more restrained (Gesto *et al.* 2013) however endocrine and behavioural effects associated with serotonergic activity are often described relative to absolute values rather than turnover of neurotransmitter to metabolite (Clotfelter *et al.* 2007; Ortega *et al.* 2013). This situation delivers something of a grey area in describing serotonin associated responses especially if all data are not presented. Unsurprisingly the use of 5OH-IAA as a suitable measurement, rather than 5-HT has however been questioned (Kiser *et al.* 2012).

In experiment 3 a trend for increased ratio of 5-HT to 5OH-IAA with increasing dietary TRP was observed as a result of a similar increase in 5OH-IAA and a stable 5-HT response. This 5-HT:5OH-IAA response may be a factor in the lack of difference between brain [TRP] as the brain TRP appears to be being utilised faster at higher dietary TRP inclusion. In experiment 4 there was a trend toward greater brain [5-HT] with increased dietary TRP inclusion at the reduced ration, yet equal brain [5HIAA] between rations. This leads to an apparent trend for a higher ratio of 5-HT to 5HIAA, or serotonergic activity in fish fed a 100% ration compared to those fed a 50% ration. No significant differences in serotonergic activity were observed between rations however supplemented feeds elevated serotonergic activity compared to controls in fish fed 100% rations.

3.5.3 Endocrine stress response

At the culmination of the 50 day experiment no differences in physiological stress response were observed between treatment for either whole body cortisol, blood glucose or blood lactate concentrations. Data reflected expected basal responses from unstressed fish and thus no physiological stress response can be attributed to long term supplementary TRP at inclusion of up to 40.9 $mg.g^{-1}$ over 50 days in juvenile barramundi. The lack of difference between treatments suggests no chronic stress response was elicited by the experimental system. Furthermore any differences in size distributions, agonistic behaviours or cannibalism specific to each tank can be ruled out as chronic stressors. These data also confirm that no acute stress response was elicited by sampling procedure.

3.5.4 Cannibalism

It doesn't appear that supplementary dietary TRP plays any role in reducing cannibalism associated mortality in juvenile barramundi, however data are presented as means and thus potentially important variations in size distributions within individual tanks are occluded. The

following summations and hypotheses must therefore be treated with caution. Survival curves of experiment 4 reveal no differences either between feed treatment or ration, and neither were dose dependent responses observed. Indeed, the feed with the poorest survival at 50% ration had the highest at 100%, while the feed with highest survival at 50% recorded the lowest at 100%. Interestingly no differences in mortality were observed between control feeds at high and low rations which is contrary to expectation as restrictions on food availability have been shown to trigger or exacerbate cannibalism directly via shortage and consequentially via differential growth (Hokanson and Lien 1986; Hecht and Pienaar 1993; Qin and Fast 1996; Jobling *et al.* 2001). When food was offered to satiation twice daily, cannibalistic associated mortality occurred within the same range as under restricted feeding in experiment 4. These data are strongly contradictory to results presented for African catfish, *Clarius gariepinus*, where feeding rates have been shown to strongly affect the rate of cannibalism. Fish which were starved, fed 5% BW.day^{-1}, fed 10% BW.day^{-1} or fed to satiation cannibalised at 100, 75, 62 and 20% respectively (Hecht and Pienaar 1993). Inadequate food supply is commonly detailed as a causative factor for increased rates of cannibalism amongst various fish species and is also suggested for barramundi, (Parazo *et al.* 1991). No evidence exists however of the impact of reduced ration on rates of cannibalism in juvenile barramundi, asides from the current experiments, which suggest that the rate of cannibalism amongst fish restricted to 50% of a satiation ration is no different to those only mildly restricted or not restricted at all. A very small difference in survival was detected in experiment 3 between feed treatments with fish fed feed 2 (19.0 mg.g^{-1}) cannibalizing conspecifics at a slower rate than in those fed the control feed (feed 1, 4.6 mg.g^{-1}). The effect was only just statistically different and, given no similar result for experiment 4 it can probably be disregarded.

The rate of food intake in juvenile barramundi fed to satiation decreases at such a rate over a size range from 30 to 124 mm that description of food intake in the standard manner as a percentage of bodyweight can provide misleading data. It is recommended that regression analysis of food intake on wet weight provides a more accurate reflection of differences for fish such as barramundi especially if significant size differences between treatments are present.

The current experiments provide conclusive evidence of dose dependent TRP induced hypophagia in juvenile barramundi from dietary inclusion as low as 14.8 $mg.g^{-1}$ (feed 2, Exp 4), with a time to reduction of ingested food ($g.day^{-1}$) at this dose of between 10 and 15 days at both rations. Time to hypophagia was displayed in a dose dependent manner. A reduction in food intake ($g.day^{-1}$) for feed containing 15.9 $mg.g^{-1}$ TRP was observed from day 5 for both rations, and on day 4 (100% ration) and day 5 (50% ration) for feed containing 19.0 $mg.g^{-1}$ TRP. Data from experiment 1 showed that higher supplementary dietary TRP inclusion elicited a faster and stronger hypophagic response in juvenile barramundi.

Though the reduction in food intake observed was clearly as a result of increased dietary TRP it remains unclear whether increased brain [5-HT] mediates the anorectic effect as serotonergic turnover appears to be self-moderated at levels outside of a normal range. It is possible that elevated levels of brain TRP, 5-HT or 5HIAA which act as precursors in other pathways, and are thus responsible for release of known hypophagic hormones such as corticotropin releasing factor (CRF). Furthermore extreme blood [TRP] may be responsible for delivering a liver or kidney response that depresses appetite. No effect of chronic stimulation of the serotonergic system was noted on the HPI axis via elevated cortisol, glucose or lactate in juvenile barramundi; however it is possible that effects were present at earlier stages in the study.

Contrary to expectation cannibalism amongst small groups of juvenile barramundi does not appear to be affected by a reduction in ration of up to 50% of satiation. Despite the literature suggesting a reduction in agonistic behaviours and cannibalism in various fish species, supplementary dietary TRP across a wide range of inclusion did not reduce the incidence of cannibalism among juvenile barramundi. Consequently the hypothesis: Supplementing fish food with TRP will reduce the occurrence of cannibalism by juvenile barramundi, compared to those fed a non-TRP supplemented feed, can be rejected for the doses and rations tested in the current studies.

4 Chapter 4 - Seawater transfer of Atlantic salmon smolts under high and low stress conditions – the effect of supplementary dietary tryptophan on growth performance and physiological stress response

4.1 Introduction

The transfer of farmed Atlantic salmon (*Salmo* salar) from a freshwater environment to a seawater environment occurs, for Tasmanian grown fish, after on average 12 months in freshwater and at a weight of approximately 120g. This transfer reflects the migration of smolts downstream at this stage in their development to spend commonly between 1 and 3 winters at sea. Most teleost species are stenohaline, and the capacity to live in both freshwater and seawater is limited to only 3 to 5% of all fish species (McCormick *et al.* 2013). This adaptation is an important and physiologically strenuous developmental event in anadromous salmonids which commonly occurs during the parr-smolt transformation or smoltification, involving substantial physiological and biochemical changes in the kidney, gut and gills, and provides increased salinity tolerance (McCormick 1996). The preparedness and the potential success of seawater transfer (SWT) is measured by plasma osmolality which describes the capacity of the fish to osmoregulate when kept in a saline water. Elevated plasma ion concentrations signify an invasion of salts and/or osmotic loss of water. Unsurprisingly, given the physiological upheaval associated with SWT, behavioural changes prevail including the onset of drinking (Usher *et al.* 1988; Fuentes and Eddy 1997), a reduction in aggressive interactions (Folmar and Dickhoff 1980), circular schooling behaviour (Folmar and Dickhoff 1980; Damsgard and Arnesen 1998) and changes to feeding behaviour which affect feed consumption (Usher *et al.* 1988, 1991; Stead *et al.* 1996; Damsgard and Arnesen 1998). Morphological changes prepare the developing salmon for a novel environment and migratory existence: the body becomes more elongate and thus more suitable for consistent swimming (Hoar 1976), and changes to eye pigmentation allow for superior vision in a saline medium via a change in retinal pigment dominance from porphyropsin to rhodopsin (Bridges and Delisle 1974; Alexander *et al.* 1994). A reduction in the

relative size of fins reflects a change in priority from station-holding to migratory swimming (Pelis and McCormick 2003), and the silvering of the skin due to the deposition of purines, specifically an increase in the guanine: hypoxanthine ratio, as by-products of protein metabolism, is thought to confer survival benefits in a pelagic environment (Hoar 1976; Folmar and Dickhoff 1980).

4.1.1 Growth performance and feeding

Interruptions to optimal food intake pose a number of problems for the fish farmer. If fish are not fed to satiation on a regular basis, problems such as the development of feeding and social hierarchies, the prevalence of agonistic behaviours with subsequent injury and wounds threatening homeostasis and providing sites for pathogens, and the variation in size at harvest, become more acute. Consequently the disruption to feeding of SWT in Atlantic salmon smolts is well documented (Usher *et al.* 1988, 1991; Stead *et al.* 1996; Damsgard and Arnesen 1998; Flood *et al.* 2011).

Usher *et al.* (1988) and (1991) found that the percentage of fish feeding post-SWT only returned to levels seen for fish retained in freshwater by approximately 33 days after transfer, while Stead *et al.* (1996) observed an overall depression in growth performance for 22 days for fish transferred to saltwater compared to previous growth performance in fresh water. Damsgard and Arnesen (1998) saw reduced feeding for saltwater transferred fish compared to freshwater retained fish at 1 week post-transfer but no differences at 4 weeks and Flood *et al.* (2011) observed higher food intake at higher feeding frequencies post-SWT. Though the depression in food intake over the period of SWT can be partially attributed to temporal physiological changes associated with smoltification, as evidenced by reduced food intake of smolts maintained in freshwater (Damsgard and Arnesen 1998), and the loss of condition factor in fish from approximately 6 weeks pre-SWT until approximately 4 weeks post-SWT (Hosfeld *et al.* 2009), in many studies the impact of transfer to an aqueous environment with a salinity greater than 20‰ is undoubtedly the trigger, though the exact mechanisms and pathways involved in the presumed appetite suppression remain unclear.

At SWT Atlantic salmon smolts have undergone physiological adaptations which permit continued osmotic control. The foremost of these, and those possibly implicated in reduced food intake are a shift in retinal pigmentation to favour shorter wavelengths more appropriate to seawater than freshwater (Alexander *et al.* 1994), chronic elevation of circulating cortisol (Specker and Schreck 1982; Young *et al.* 1989; Franklin *et al.* 1992; McCormick *et al.* 2007; Ebbesson *et al.* 2008), up-regulation of the serotonergic system (Ebbesson *et al.* 1992; Ebbesson *et al.* 1996; Ebbesson *et al.* 2003), a change to the adult isoform of haemoglobin (Koch 1982), an increase in buoyancy (Saunders 1965), the onset of drinking (Usher *et al.* 1988; Fuentes *et al.* 1996; Fuentes and Eddy 1997) and an increased salinity tolerance via increased gill Na+, K+ -ATPase activity, the number and size of gill chloride cells and intestinal water permeability (Boeuf 1993). The duration of interruption to feeding at SWT in Atlantic salmon smolts has been variously reported to range from as little as a couple of days (Boeuf 1993), 11 days (Flood *et al.* 2011), 22 days (Stead *et al.* 1996), 30 + days (Jørgensen and Jobling), 5 weeks (Usher *et al.* 1991), 8 weeks (Stradmeyer 1994) to no resumption in the case of 'pinheads' or 'failed smolts'.

4.1.2 Physiological stress response

Stress is a loosely used term and even within biological contexts stress, stressors and stress responses have relatively non-finite boundaries in their description. Within the context of this thesis stress is defined as the disturbance of homoeostasis, the self-regulating process by which biological systems maintain stability while adjusting to conditions that are optimal for survival, in response to extrinsic or intrinsic stimuli, or stressors. Stressors in teleost fish are described as producing a set of coordinated behavioural and physiological responses which are categorized in numerous ways (Wendelaar Bonga 1997). Persistent behavioral responses such as subordination or reduced food intake, while reliable indicators of underlying conditions, are most likely mediated via a specific or integrated endocrine response. Disruption to homeostasis delivers what has been categorized as a three tier response: primary, a neuroendocrine release of catecholamines and corticosteroids along with fight or flight; secondary, physiological changes including increase of red blood cells and elevation of circulating glucose and

behavioural changes such as colouration or reduced feeding, and tertiary, long-term whole animal responses such as depressed growth and reproductive performance and elevated occurrence of disease and mortality (Ellis *et al.* 2012).

There are many hormones or precursor hormones associated with the stress response in fish such as corticotropin releasing hormone (CRH), adrenocorticotropin hormone (ACTH), cortisone, epinephrine, and norepinephrine. However cortisol concentrations in the blood have become the standard measure of hypothalamus pituitary interrenal (HPI) axis activation, as sampling and processing are relatively simple, and the response to stimulus reasonably delayed, yet identifiable, for both acute and chronic stressors. In the current study the measure used to describe the level of stress response is serum cortisol concentration.

Standard husbandry practices have been shown to deliver physiological stress responses that depress growth performance via both restraint of feeding behaviour and suppression of appetite (Pickering 1993; Schreck C. B. *et al.* 1997; McCormick *et al.* 1998; Bernier and Peter 2001b; Bernier 2006). Furthermore, fish subjected to stressors have been shown to have a reduced feed conversion efficiency (Barton *et al.* 1987b; Gregory and Wood 1998) via disrupted or increased metabolic activity and / or the reduction of intestinal absorption (Barton *et al.* 1987b; Mommsen *et al.* 1999).

The primary focus of research into the physiology of the stress response in fish has been to confirm HPI axis activation under varying stimuli and quantify the stress response as a measure of circulating cortisol. Elevated circulating cortisol has been described during the entire period of smoltification with a brief but large peak at SWT (Specker and Schreck 1982; Barton *et al.* 1985; Young *et al.* 1989). Hypothalamic CRH activation and subsequent cortisol production should not necessarily be adversely viewed; at smoltification benefits are conferred from elevated cortisol as treatment with cortisol prior to SWT enhances hypo-osmoregulatory capacity in salmonids (Madsen 1990; Fuentes *et al.* 1996). Unsuccessful transfers of salmon to saltwater have resulted in the transfer-associated peak being maintained, probably as a result of failed homeostatic mechanisms (Franklin *et al.* 1992). Thus circulating cortisol appears to well define the cumulative stressors associated with smoltification and SWT. Elevated cortisol is

implicated in numerous conditions of concern to commercial aquaculture: feeding depression, reduced locomotor activity, modified shoaling / schooling, poorer FCR, greater size variance, reduced reproductive performance and increased incidence of disease and mortality (Ellis *et al.* 2012).

4.1.3 Smolt Behaviour

Given the importance of salmon within the global aquaculture industry and the impact on growth performance and productivity from adverse behaviour, it is not surprising that there is a substantial body of work addressing behaviour amongst the salmonids. Underlying behavioural changes, associated with the process of smoltification and the migration from freshwater to seawater, as previously described, were taking place, and thus were open to manipulation via elevated dietary TRP. Though this study was not recording behaviour *per se*, food intake was being measured as a proxy for feeding behaviour of a group of fish relative to dietary and SWT treatment. Many of the behaviours exhibited at this time are synonymous with reduced food intake, and reduced food intake is a commercially relevant measure. In light of the importance of behavioural elasticity at this time of development and to the outcome of this experiment, it is apt to present a brief audit of the relevant behavioural literature.

Anadromous salmonids most likely evolved in freshwater and developed the capacity of saltwater tolerance. It has been suggested that downstream migration, and subsequent requirement of euryhalinity, may have been driven by behaviour (Hoar 1976); For example Atlantic salmon display more frequent aggressive interactions when food is scarce (Symons 1968), and variation in growth in chum salmon is more limited, and attributed to fewer aggressive interactions, for those fed a greater ration (Davis and Olla 1987). More recent laboratory studies suggest no differences in rates of aggressive interactions between parr and smolts (Damsgård and Arnesen 1998). It is however possible that previously observed reductions in aggressive interactions came as a result of sibling grouping in wild fish; sibling Atlantic smolts are more likely to migrate together, thus showing a degree of kin recognition (Olsen *et al.* 2004). A further study on the kinship of migrating Atlantic salmon smolts also found a reduced time gap between migrating siblings, however found no evidence for familial

schooling (Fernandes *et al.* 2015). The downstream migration features a distinct change from territorial to schooling behaviour and it is suggested that this seaward passage is assisted by changes in buoyancy , associated changes to position in the water column, and seasonally elevated water velocity (Saunders 1965; Gibson 1983). Further impacts on the ability to maintain position within the water flow result from reduced pectoral, pelvic and adipose fin lengths relative to fork length in Atlantic salmon smolts compared to parr of a similar size (Pelis and McCormick 2003). A reduction in locomotor activity at this time probably also contributes to downstream passage. With downstream migration comes increased salinity triggering a surge in drinking behaviour for the marine-bound smolt and this ingestion of seawater, desalinated in the oesophagus, compensates for loss to the hyperosmotic environment. Within 5 days seawater is being imbibed at approximately 6.5 $mL.Kg.h^{-1}$, and this rate is not affected by the amount of ingested food in the stomach (Usher *et al.* 1988; Fuentes and Eddy 1997).

4.1.4 Salmonid tryptophan research

Feed costs represent the single greatest running cost for many finfish production companies, thus the desire for premium feeds containing high quality protein with the appropriate amino acid composition for the animal in question is high. High quality protein can be described by the digestibility and availability of the amino acids (Rollin *et al.* 2003), and the degree to which the absorbed amino acid mix accords with the requirements of the animal (Wang and Fuller 1989). Surprisingly, indispensable amino acid requirements for many cultured species are not readily available in the literature. The requirement for dietary inclusion of amino acids has been explored in Atlantic salmon fry (Rollin *et al.* 2003). Studies of this nature aim to pinpoint the appropriate amino acid balance for optimal growth. Vertebrates use protein for tissue synthesis, however fish also utilize amino acids extensively for energy. Furthermore amino acids are regulators of gene expression and the protein phosphorylation cascade, as well as precursors of hormones with significant biological importance (Wu 2009). The previous author presents a list of metabolites and functions of amino acids in nutrition and metabolism in vertebrates. The physiological functions and pathways of TRP in fish have been presented in the

general introduction, however it seems pertinent to review the literature surrounding supplementary dietary TRP and salmonids.

The indoleamine serotonin is, like the catecholamines (dopamine, norepinephrine and epinephrine) a monoamine neurotransmitter and the organization of monoaminergic systems has been shown to be very consistent across all vertebrates and seems to have occurred early in phylogeny (Parent 1984). TRP and tyrosine, the precursor aromatic amino acids of serotonin and the catecholamines respectively, both compete for transport via a common carrier across the blood brain barrier alongside other large neutral amino acids (Fernstrom and Fernstrom 2007). Concentrations of circulating and brain TRP depend on levels of dietary TRP (Lepage *et al.* 2002).

Studies using supplementary dietary TRP on salmonids have shown altered behavioural, feeding, and endocrine stress responses. The number of aggressive acts perpetrated by a resident rainbow trout against an intruder were reduced in fish fed supplementary dietary TRP for 7 days (Winberg *et al.* 2001; Lepage *et al.* 2005). No differences in food intake were observed when rainbow trout were fed TRP supplemented feed at doses up to 74.3 $g.Kg^{-1}$ for between 3 and 98 days (Johnston *et al.* 1990; Winberg *et al.* 2001; Lepage *et al.* 2002; Lepage *et al.* 2003; Lepage *et al.* 2005), furthermore food intake and feed efficiency (FCR) were seen to increase in fish fed TRP supplemented feed at 26.9 $g.Kg^{-1}$ despite depressed SGR (Papoutsoglou *et al.* 2005). Conversely a negative correlation between meal size and 5HIAA/5HT ratio has been presented (Winberg *et al.* 1993; Alanärä *et al.* 1998), which more closely reflects the sizeable body of literature describing appetite suppressing effects of an up-regulated serotonergic system on mammals (Blundell and Halford 1998). A short term anorectic effect of fenfluramine, a serotonin releasing agent, administration has been observed in rainbow trout (Ruibal *et al.* 2002). Perhaps surprisingly, given the observed anorectic effect of faster turnover of serotonin, food deprived fish have been shown to have a higher 5HIAA/5HT ratio (Ruibal *et al.* 2002), however no such effect was observed in starved Arctic char (Winberg *et al.* 1992). HPI axis activation has been linked with both catecholaminergic and serotonergic activity in salmonids (Winberg and Nilsson 1993a). Administration of TRP supplemented feed delivered an increased

cortisol response in unstressed fish and a reduced cortisol response in stressed fish (Lepage *et al.* 2002).

The stress response in fish, as described, delivers a coordinated neuroendocrine, physiological and behavioural response, which on a simplistic timeline begins in the brain and ends, if the stressors are not removed, with the discussed adverse behaviours and negative performance measures. The association between the HPI regulated stress response and the brain serotonergic system has been well documented, so much so that fluctuations in serotonergic activity have been used to describe stress responses in rainbow trout (Gesto *et al.* 2013; Fraser *et al.* 2015). The entire period of smoltification in Atlantic salmon is characterized by reduced food intake and chronic physiological stress response, with both parameters at their most extreme at SWT.

Given the close association between the HPI axis and the serotonin pathway, the known stressors and stress responses observed at SWT for farmed Atlantic salmon, and the effect of dietary TRP on brain TRP and consequent activation of the serotonergic system, an experiment was designed to answer the question: Does supplementary dietary TRP reduce feeding depression at SWT via suppression of cortisol response? More specifically, does it have an effect on either growth performance (measured as food intake, length gain, weight gain, FCR and SGR), stress physiology at 24 h post-transfer (measured as serum cortisol concentration), or readiness for transfer, as indicated by serum osmolality, in Atlantic salmon smolt at SWT subjected to either high or low stress SWT, and fed one of four feeds containing TRP at 4.9 $mg.g^{-1}$, 10.9 $mg.g^{-1}$, 21.8 $mg.g^{-1}$ or 46.3 $mg.g^{-1}$.

- Hypothesis: Supplementing fish food with TRP will increase food intake in Atlantic salmon smolt at SWT via moderation of HPI axis response compared to those fed a non-TRP supplemented feed

Specifically this study aims to:

- Assess whether supplementary dietary TRP moderates the feeding depression observed in Atlantic salmon smolts at SWT.
- Assess whether feeding depression commonly observed in Atlantic salmon smolts at SWT can be attributed to transfer type, ie. high stress or low stress.
- Examine whether supplementary dietary TRP affects the physiological stress response, as indicated by serum cortisol concentrations, in Atlantic salmon smolts at SWT.
- Examine whether the physiological stress response at 24 h post-transfer is affected by transfer type, ie. high stress or low stress.
- Examine whether supplementary dietary TRP mediates serotonergic activity in Atlantic salmon smolt

4.2 Materials and Methods

4.2.1 Fish and experimental system

The experiment was conducted at the IMAS Aquaculture Centre within the University of Tasmania's Launceston campus. Female diploid salmon were due to be sourced from the Petuna hatchery in Cressy, Tasmania however due to limited stock at this site fish were sourced from the Tassal's Rookwood Rd. hatchery at Ranleigh, Tasmania. These fish were all female triploids which were not going to be utilized further by Tassal and exhibited a high occurrence of lower jaw deformity. Eight hundred fish were transported to the IMAS Aquaculture Centre by road under light AQUI-S sedation and with the use of pure oxygen. On arrival fish were transferred into a 3500 L freshwater holding tank at 14°C and appropriate filtration for environmental acclimation prior to sorting for length, weight and health indicators (scale loss, fin damage, spinal and jaw deformities) for experimental inclusion. Two weeks after arrival (7th august 2013) each experimental tank was stocked with 31 fish with an average length and weight of 19.34 cm ± 0.81 cm, and 80.08 g ± 0.44 g respectively. Fish were fed a 3 mm pelleted feed (Nutra Supreme, Skretting, Cambridge, Tasmania) during the acclimation period and the pre- experimental feeding phase (days 1 – 13) and were considered acclimated when all fish were feeding and tank average daily food intake reached 1% of body weight. Five weeks after arrival (26th August 2013) acclimated fish were anaesthetized, re-measured (19.96 cm ± 0.83 cm) and re-weighed (86.92 g ± 0.47 g) and the 27th August was considered day 1.

A recirculating system was constructed comprising 24 grey flat based 300 L tanks, 1.5 mm plastic screens and FSI 100µ filter bags for large particulate removal, foam fractionation for small (< 30 µ) particulate removal, biofiltration media, UV irradiation (2 x 50W Emperor Aquatic, Pottstown, PA, USA) for bacteriological control and two heat/chillers (Carrier, Dingley, Victoria, Australia) for maintaining water temperature at 12°C. Two Onga 413 pumps combined to deliver a directional flow of 10.5 L.m^{-1} to each tank, giving 2.1 fold turnover of tank volume each hour and sufficient centripetal force to deliver faecal waste and uneaten feed to the centre of the tank within 2 m. Effluent water was extracted from the tank base at 53 cm.s^{-1} via a 20 mm diameter PVC pipe which passed through the tank wall 8 cm below the top of the tank.

By this method uneaten feed was extracted from the tank within 2 minutes. A 1 mm screen trapped faecal matter and uneaten feed for quantitation post-feeding before tank waters were amalgamated for further finer particulate removal and biofiltration. Tanks were fitted with netting covers which allowed for good observation of fish, light penetration and feeding however prevented aerial escape. Aeration was provided using airstones attached to the centre-most point of the effluent pipe. Ambient light entered via opaque overhead skylights however photoperiod was maintained by fluorescent lighting at 24:0 L:D throughout acclimation and up until seawater transfer to assist with smoltification. This photoperiod was continued through final sampling to eliminate this factor as a potential cause of food intake interruption. Throughout acclimation and up until saltwater transfer on day 21 water from the municipal water supply was added at an approximate rate of 10% of total system volume per day. After saltwater transfer water was batch exchanged on a weekly basis with an average salinity during this phase of 30.6 ± 0.9 ‰.

4.2.2 Fish Feeds and feeding

Over the course of the experiment fish were fed 3 mm pelleted commercial Atlantic salmon feeds or 3mm experimental feeds. Nutra Supreme (Skretting, Cambridge, Tasmania) was used as an acclimation feed and as the base ingredient of experimental feeds up until saltwater transfer on day 21, while Spirit Supreme (Skretting, Cambridge, Tasmania) was used as a post-seawater transfer-feed (feed from day 38 until the end the end of the experiment on day 45), and as the base ingredient of the post-seawater transfer experimental feeds between day 22 and day 37. To clarify reporting the 45 day experimental period was split into 5 phases. See Table 4.1 (p 142).

Table 4.1 Experimental phases detailing duration, salinity and feed type.

Phase	Days	Salinity (ppt)	Feed
1	1 - 13	0	Pre-transfer commercial feed
2	14 - 20	0	Pre-transfer experimental feed with supplementray TRP
3	21 - 23	20 - 30	Post-transfer experimental feed with supplementray TRP
4	23 - 38	31	Post-transfer experimental feed with supplementray TRP
5	39 - 45	31	Post transfer commercial feed

Four experimental feeds (1 reference and 3 with supplementary TRP) were made by hammer milling the appropriate commercial feed, Nutra Supreme during the freshwater phase and Spirit Supreme during the saltwater phase, then re-pelleting the mixture through a 3 mm die after adding the required amount of TRP, balancing the supplementary TRP with α-cellulose and using carboxy methyl cellulose (CMC) as a binding agent. See Table 4.1 (p 142).

Table 4.2 Composition of feeds fed to Atlantic salmon smolts throughout a 45 day period over saltwater transfer. Feeds were created using a Skretting salmon feed (*Nutra Supreme and ¤Spirit Supreme) as a base with supplementary tryptophan, balanced with α-Cellulose and using CMC as a binder. The mix was extruded through a 3mm die.

	Pre SW Transfer					Post SW Transfer				
Ingredient composition (mg.g^{-1}) DM	Commercial feed*	Feed 1	Feed 2	Feed 3	Feed 4	Commercial feed¤	Feed 1	Feed 2	Feed 3	Feed 4
Base commercial feed	1000	930.5	930.5	930.5	930.5	1000	930.5	930.5	930.5	930.5
CMC	n/a	20	20	20	20	n/a	20	20	20	20
α-Cellulose	n/a	49.33	42	28.2	0	n/a	49	42	28.01	0
Tryptophan	n/a	0.57	7.23	21.34	49.62	n/a	0.49	7.49	21.49	49.49
TOTAL	1000	1000.4	999.7	1000.0	1000.1	1000.0	1000.0	1000.0	1000.0	1000.0
Water (mL. Kg^{-1})	n/a	216.6	100	116.6	116.6	n/a	116.6	133.3	133.3	108.3
Pellet weight (mg)										
	39.7 ± 1.09	32.7 ± 0.35	31.8 ± 0.40	35.8 ± 0.43	36.2 ± 0.63	37.7 ± 0.94	33.4 ± 0.29	34.4 ± 0.44	32.9 ± 0.29	35.6 ± 0.57
Liquid hydrolysis + UPLC (mg.g-1) DM										
Tryptophan	4.7	4.9	10.3	23.8	44.5	5.3	4.9	10.9	21.8	46.3
Chemical composition (%)										
Crude protein (%)	48.0	47.1	48.2	48.9	51.7	50.7	48.2	49.0	50.8	51.4
Crude lipid (%)	19.2	20.1	20.1	20.0	20.4	22.7	22.2	21.7	21.3	21.6
Moisture (%)	7.5	7.2	7.3	7.0	6.8	8.5	5.8	5.9	5.3	6.6
Ash content (%)	7.6	7.5	7.6	7.7	8.2	8.0	7.6	7.8	8.0	8.1

Fish were fed twice daily, in the morning between 08:00 and 10:00 h and in the afternoon between 17:00 and 19:00 h. Feeding was conducted by hand and each tank was visited twice during each feeding session with fish being fed until they ceased to ingest feed and waste pellets were observed on the screens. After each round of feeding, uneaten feed was counted and the number of pellets multiplied by the average pellet weight. Food intake was calculated as *feed ration – feed waste = food intake (g.day^{-1})*, and presented as daily consumption as a percentage of body weight. The four feeds and two methods of saltwater transfer (section 4.2.3) delivered eight treatments in triplicate.

4.2.3 Seawater transfer treatments and sampling procedures

Two different methods of saltwater transfer were conducted with one being described as a low stress (L/S) transfer in which tanks were flooded with seawater until salinity reached 30 ppt, and the other being described as a high stress (H/S) transfer where freshwater was drained until the backs of the fish were exposed and equilibrium became compromised. This situation was maintained for 5 minutes prior to filling with pre-prepared seawater at 30 ppt. The addition of sea salt was used to counteract the presence of fresh water in the RAS. An unexpectedly slow rate of dissolving retarded the maintenance of 30 ppt to both transfer types for two days around SWT.

Individual lengths and weights were recorded at the beginning of the experiment, at saltwater transfer, and at the end of the experiment. Immediately prior to saltwater transfer on day 21, two days after saltwater transfer on day 23, and at the end of the experiment 5 fish from each tank were randomly selected, anaesthetized (18.75mg.L^{-1} AQUI-S, New Zealand), and measured for weight (g) and fork length (mm). A blood sample was taken and stored on ice before the fish was euthanized via severance of the spinal cord, and the cranium was opened dorsally with a downward scalpel incision toward the mouth for excision and the brain removed and immersed in liquid nitrogen. The removal and freezing of the brain took less than 90 seconds. Brains were transferred to -80° C storage. Blood samples were centrifuged at 1500g for 10 minutes and the

serum was quickly pipetted into two 1.5 mL Eppendorf tubes and frozen at -80° C in preparation for osmolality and cortisol measurement.

4.2.4 Osmolality and endocrine response

Measurements of serum osmolarity (mmol.mL) were made using a Vapro© Vapor Pressure Osmometer, model 5520, (Wescor Inc, Logan, UT, USA) to determine whether the Atlantic salmon smolts were physiologically ready to undergo SW transfer, and to assess whether supplementary dietary TRP or transfer technique affected serum osmolality.

Serum cortisol was determined via an enzyme immunoassay (EIA) kit (Cortisol EIA Kit, Catalog No. ADI-901-071, Enzo Life Sciences) which is a competitive immunoassay for the quantitative determination of cortisol in biological fluids.

4.2.5 Brain Tissue Sampling

4.2.5.1 Quantitation

A novel method for quantitation of the desired analytes from fish brains was developed in conjunction with David Nichols of Central Science Laboratory, UTAS, based on a method for brain neuroendocrine determination in rat brain (Park *et al.* 2013). A stable isotope dilution was used for quantitation of TRP and neuroendocrine analytes 5HT and 5OH-IAA, with the addition of deuterated surrogate standards to excised brain tissue prior to homogenization and treatment. To further stabilize the sample and improve detection a derivatisation was used. This simple robust step could proceed rapidly at room temperature in the aqueous sample matrix, and the resultant ethyl chloroformate derivatives could be extracted from the aqueous mixture by organic solvent, thereby providing an additional purification step from the sample matrix. Ultra performance liquid chromatography (LC) coupled with triple quadrupole tandem mass spectrometry (MS/MS) for detection and quantitation were experimentally determined and optimised using analytical standards of both native analytes and deuterated labeled compounds. Detection sensitivity was determined to be approximately 1pg.

4.2.5.2 Brain sample preparation

Each fish brain was removed from -80° C storage and quickly weighed to the nearest µg in a 2 mL Eppendorf tube. The tube was immersed in ice and 100 µL of the spiking standard of 2µg.mL^{-1} of deuterated TRP, 5HT and 5OH-IAA was added. Sixty seconds later a fourfold volume of ice cold 0.1 M H_2SO_4 was added and the contents were sonicated using a Microson Ultrasonic Cell Disruptor (Microsonix Inc., Farmingdale, NY, USA) for 5 seconds at 5W. Homogenates were centrifuged (Eppendorf Centrifuge 5810R) for 15 minutes in a chilled centrifuge (4°C) at 16,600g. Supernatant was aspirated and identical centrifugation was repeated. Supernatant was transferred to a 0.5 mL Eppendorf tube and stored for derivatisation at -80°C.

4.2.5.3 Derivatisation

A volume of 185 µL of thawed, ice cold brain homogenate solution was transferred to an 8 mL glass reaction vial (Cat # 98008, Grace Discovery) immersed in ice. To the vial was added 265 µL of ice cold distilled water, 300 µL of a 4:1 ethanol:pyridine solution and 20 µL of ethyl chloroformate before capping the vial and shaking for 4 minutes. To this was added 1000 µL of diethyl ether prior to freezing for 2 hours at -80^{o}C or until the aqueous layer was frozen. After removing from -80^{o}C a Pasteur pipette was used to remove the upper organic layer to an HPLC vial (Cat # 95191 Grace Discovery) and stored at -80^{o}C in preparation for HPLC.

4.2.5.4 Brain analyte quantitation

The derivatised samples were analyzed using a Waters Acquity H-Class Ultra Pressure Liquid Chromatography (UPLC) instrument coupled to a Waters Xevo triple quadrupole mass spectrometer. A Waters Acquity UPLC BEH C_{18} column (2.1 mm × 100 mm × 1.7 µm) was used. The mobile phase consisted of two solvents: 1% (v/v) formic acid in water (solvent A) and acetonitrile (solvent B). The UPLC program was 97% A:3% B to 100% B at 6.0 min, which was held for 1 min, and this was followed by immediate re-equilibration to starting conditions for 3 min. The flow rate was 0.30 mL min^{-1}, the column was held at 35°C, and the sample

compartment at 6°C. Injection volume was 1 uL. Approximate retention times are listed in Table 2.

The mass spectrometer was operated in positive ion electrospray mode with a needle voltage of 2.9 kV, and multiple reaction monitoring (MRM) was used to detect all analytes. See Table 4.3 (p 147). Dwell time for each MRM transition was 18 msec. Cone voltages and collision energies were optimized for each MRM transition as described in Table 2. The ion source temperature was 130°C, the desolvation gas was N_2 at 950 L h^{-1}, the cone gas flow was 100 L h^{-1}, and the desolvation temperature was 450°C. Data were processed using MassLynx software.

Table 4.3 Mass spectrometer multiple reaction monitoring operational data for the analytes serine, 5 OH IAA, TRP and their respective deuterated isotopes.

Analyte	Retention time (min)	Precursor (m/z)	Quantitation product (m/z)	Qualification product (m/z)	Cone voltage (V)	Collision voltage (V) (Quant/Qual)
Serine		321.2	160.1	203.2	23	28 / 17
D4-Serine	4.57	325.2	164.1	207.2	23	28 / 17
5-OH-IAA		292.2	218.2	146.1	35	13 / 33
D5-5-OH-IAA	4.82	297.2	223.2 + 222.2	151.1	35	13 / 33
Tryptophan		305.3	231.2	259.2	30	15 / 11
D5-Tryptophan	4.74	310.2	236.2 + 235.2	264.2	30	15 / 11

4.2.6 Calculations

Feed conversion ratio (FCR) was calculated as:

FCR ($g.g^{-1}$) = Weight of ingested feed (g) / Weight gain (g)

Condition factor (K) was calculated as a measure of individual condition within and between treatments. These data were used to eliminate from further analysis two fish, which showed very low k from lack of food intake, one each from feeds 3 and 4 which didn't resume feeding post-saltwater transfer.

Condition factor was calculated as:

$$K = 100 \text{ [Wet weight (g) / Fork length (cm)}^3\text{]}$$

Specific growth rate (SGR) was calculated as:

$$SGR (\% d^{-1}) = 100 ((\ln W_t - \ln W_i)/t)$$

where W_i and W_t are initial and final weights respectively and t is time (days) between initial and final weighing.

The coefficient of variation (CV) was calculated as:

$$CV = 100 \text{ (standard deviation / mean)}$$

4.2.7 Statistical analysis

Statistical analyses were performed using SPSS version 21 (SPSS, 2014) and GraphPad Prism version 6.0 for Windows, GraphPad Software, La Jolla California USA. Mean values are reported ± standard error of the mean (SEM). All distributions were found to be normal by the D'Agostino & Pearson omnibus normality test. Homogeneity of variances was tested graphically by examination of residual plots in SPSS and by the Brown-Forsythe test. Data were tested for differences between treatments using one way and two way ANOVA, or independent samples *t*-tests and multiple comparisons were made using Tukey and Bonferonni tests. Differences were considered significant at $p < 0.05$.

4.3 Results

4.3.1 Food intake and growth

Over days 1 to 13 (phase 1) whilst all fish were being fed a commercial feed there were no differences in food intake between treatments. Over days 14 to 20 (phase 2) whilst fish were being fed experimental feeds containing supplementary TRP a difference in food intake between feeds was observed ((F = 10.18, *df* 3, 24, $p < 0.001$) with feed 4 being consumed at a lower rate (in terms of $\%BW.d^{-1}$) than the other feeds. There was no difference in food intake for feeds 1, 2 and 3 between phase 1 and phase 2 (14 to 20) whereas with feed 4 there was a reduction in food intake. A reduction in food intake was observed for all feeds following SWT over days 21 to 23 (phase 3) when compared to phase 2. When comparing the 7 days pre-SWT (phase 2) to the 7 days post-SWT there was a reduction in food intake for all feeds (F = 33.56, df 7, 48, $p < 0.0001$). Following SWT during days 24 to 37 (phase 4) while fish were being fed experimental feeds containing supplementary TRP there were no differences in food intake between feeds nor were there differences in food intake between phase 4 and phase 2. Feed was consumed at a greater rate (in terms of $\%BW.d^{-1}$) in all the groups in phase 5 (days 38 to 45) than in phase 4 (F = 12.55, df 7, 80, $p < 0.0001$) however these differences were mainly accounted for by the first 7 days of phase 4 where food intake for all feeds was less than for phase 5 (F = 49.42, df 7, 52, $p < 0.0001$). Only feed 4 was consumed at a greater rate (in terms of $\%BW.d^{-1}$) in phase 5 than during the last 7 days of phase 4 ($p < 0.05$). Feed was consumed at a greater rate (in terms of $\%BW.d^{-1}$) in all the groups during phase 5 than during phase 1 (F = 12.56, df 7, 76, $p < 0.0001$).

SWT accounted for a difference in food intake for feed 2 during phase 4. See Figure 4.2 (p 153). There was no difference in food intake between SWT methods for any other feeds.

Over the full course of the experiment no differences in food intake ($g.fish.d^{-1}$) were observed between SWT treatments (Fig 1). A significantly lower feed intake was recorded for feed 4 when compared to feed 1 (F = 1.31, df 44, 44; $p < 0.05$) (Figure 4.1, p 152).

Mortality, excluding fish removed for sampling, was less than 1% and no differences in survival among treatments were observed. See Table 4.4 (p 160). No differences were observed for either weight or length of any treatment at the beginning of the experiment, pre-SWT sampling, post-SWT sampling or when data from pre and post-SWT samplings were pooled. The final weights of fish being fed feed 4 was less than the final weights of those fed feed 1 however no other differences in final weight were observed. See Table 4.4 (p 160). Increase in biomass was found to be lower for those fish fed feed 4 than for those fed all other feeds. No differences in final lengths were observed however length gain of those fish fed feed 4 was significantly less than those fish fed feed 1. See Table 4.4 (p 160). Despite differences in feed intake over the course of the experiment no differences in FCR were observed between feed treatments or SWT method (Figure 4.3, p 154), however SGR (%.d^{-1}) for feed 4 was lower than for the other 3 feeds. See Figure 4.4 (p 154). Differences in SGR between SWT methods were not observed. Condition factor (k) improved for all feeds over the course of the experiment except for feed 4, however SWT method did not affect k. See Figure 4.5 (p 155).

4.3.2 Osmolarity and endocrine

SWT was found to have a significant effect on serum cortisol (ng.mL^{-1}) concentrations which were greater post-SWT than pre-SWT. No differences in serum cortisol were observed between SWT method however. See Figure 4.6 (p 156).

Both feed and transfer type had a significant effect on serum osmolality (mM.mL^{-1}) however the effect of feed type was not strong and differences were not identifiable by Tukeys HSD. The effect of transfer type was extremely significant and was higher in fish subjected to HS SWT compared to fish subjected to LS SWT for all feed treatments. See Figure 4.7 (p 157).

4.3.3 Neuroendocrine

Brain TRP (µg.g brain^{-1}) was different for each of the four feeds pre-SWT (Figure 4.8, p 158) and related to TRP content of feed. See Table 4.2 (p 143) . Differences were observed between all feeds except feeds 1 and 2 post-SWT however no differences between feeds were observed at final sampling. See Figure 4.8 (p 158). Temporal analysis between sampling times showed no differences in brain [TRP] for feed 1, a reduction at final sampling compared with pre- or post-SWT sampling for feed 2, and a reduction over time for feeds 3 and 4. A difference in brain [TRP] was observed at post-transfer sampling between transfer types (F 4.25, *df* 2, 26, $p < 0.05$) (Figure 4.9, p 159).

There were no differences in brain serotonin (ng.gbrain^{-1}) between feeds at any timepoint (Figure 4.8, p 158) however a reduction was observed for all feeds except feed 3 between the pre-SWT sampling and the final sampling.

Brain 5OH-IAA (ng.gbrain^{-1}) was found to increase relative to TRP content of the feed. See Figure 4.8 (p 158). Differences were observed between all feeds except feeds 1 and 2 pre-SWT. Post-SWT brain 5OH-IAA for fish fed feed 1 was different to those fed feeds 3 and 4, and fish fed feed 2 had different brain 5OH-IAA compared those fed feed 4, however no differences were observed between fish fed feed 2 and fish fed either feed 1 or feed 3. No differences were observed between feeds at the final sampling. Temporal analysis between sampling times showed no differences in brain 5OH-IAA for feed 1, a reduction at final sampling compared with pre- or post-SWT sampling for feed 2 and feed 3, and a reduction over time for feed 4.

The ratio of 5OH-IAA: 5HT in the brain was found to increase relative to an increase in TRP content of the food. See Figure 4.8 (p 158). Differences were observed between all feeds except feeds 1 and 2 pre-SWT. Post-SWT ratio of 5OH-IAA: 5HT in the brain for fish fed feed 1 was different to those fed feed 4, and fish fed feed 2 had different brain 5OH-IAA compared those fed feed 4. No differences were observed between feeds at the final sampling. Temporal analysis showed an increase in the ratio of 5OH-IAA: 5HT in the brain for fish fed feed 1 at final sampling when compared to either pre or post-SWT. There were no other differences between timepoints for any other feed.

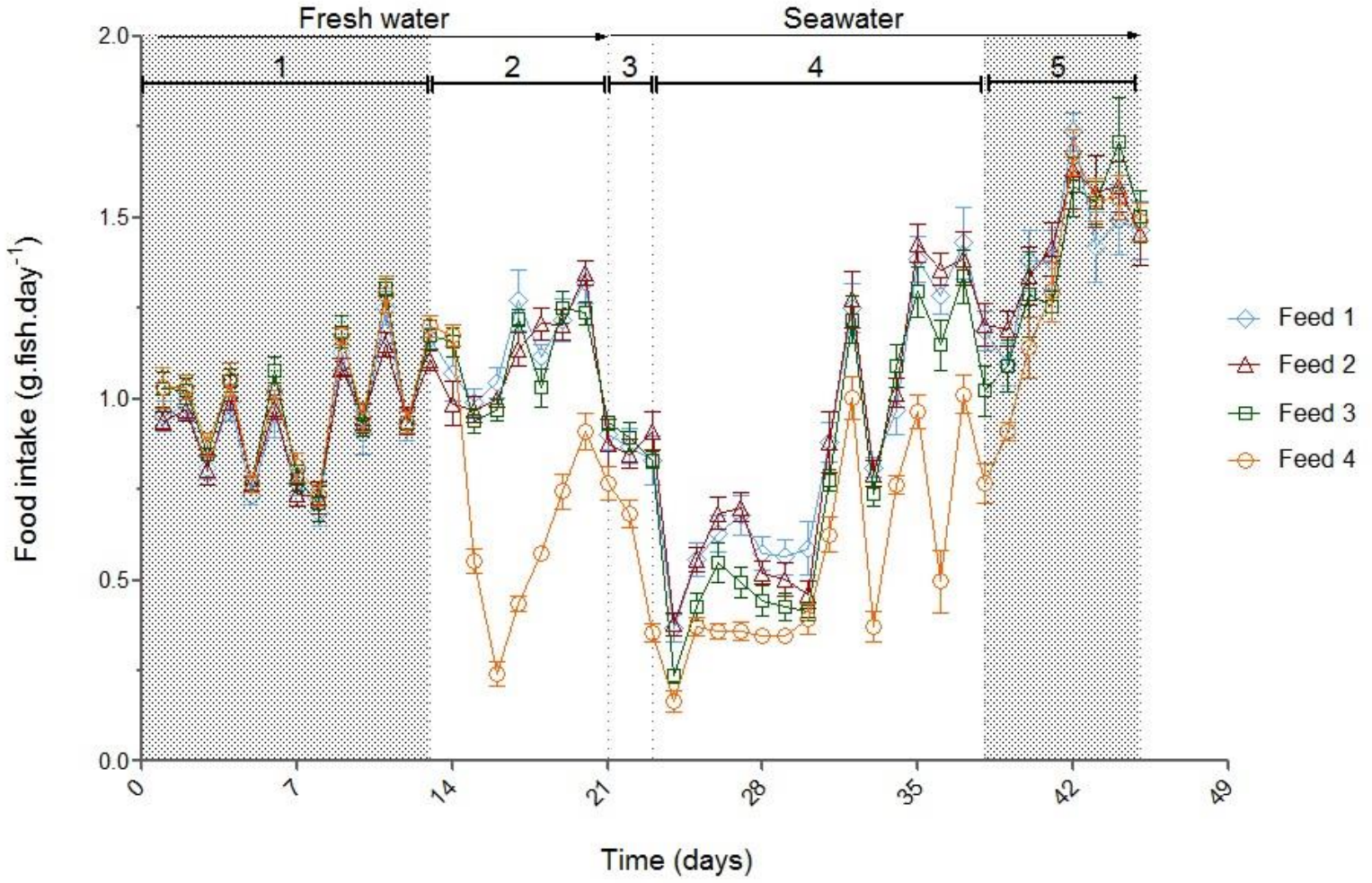

Figure 4.1 Mean food intake ± SEM pooled across low stress and high stress SW transfer presented as g.fish^{-1}.day^{-1} over 45 days (subdivided in to 5 phases) in freshwater and seawater (30 ‰) whilst on commercial feed or modified feeds with supplementary TRP at inclusion of: Feed 1, 4.9 mg.g^{-1}; Feed 2, 10.9 mg.g^{-1}; Feed 3, 21.8 mg.g^{-1}; and Feed 4, 46.3 mg.g^{-1}. Food intake for feeds 1 and 2 were found to be greater than for feed 4 ($p < 0.05$).

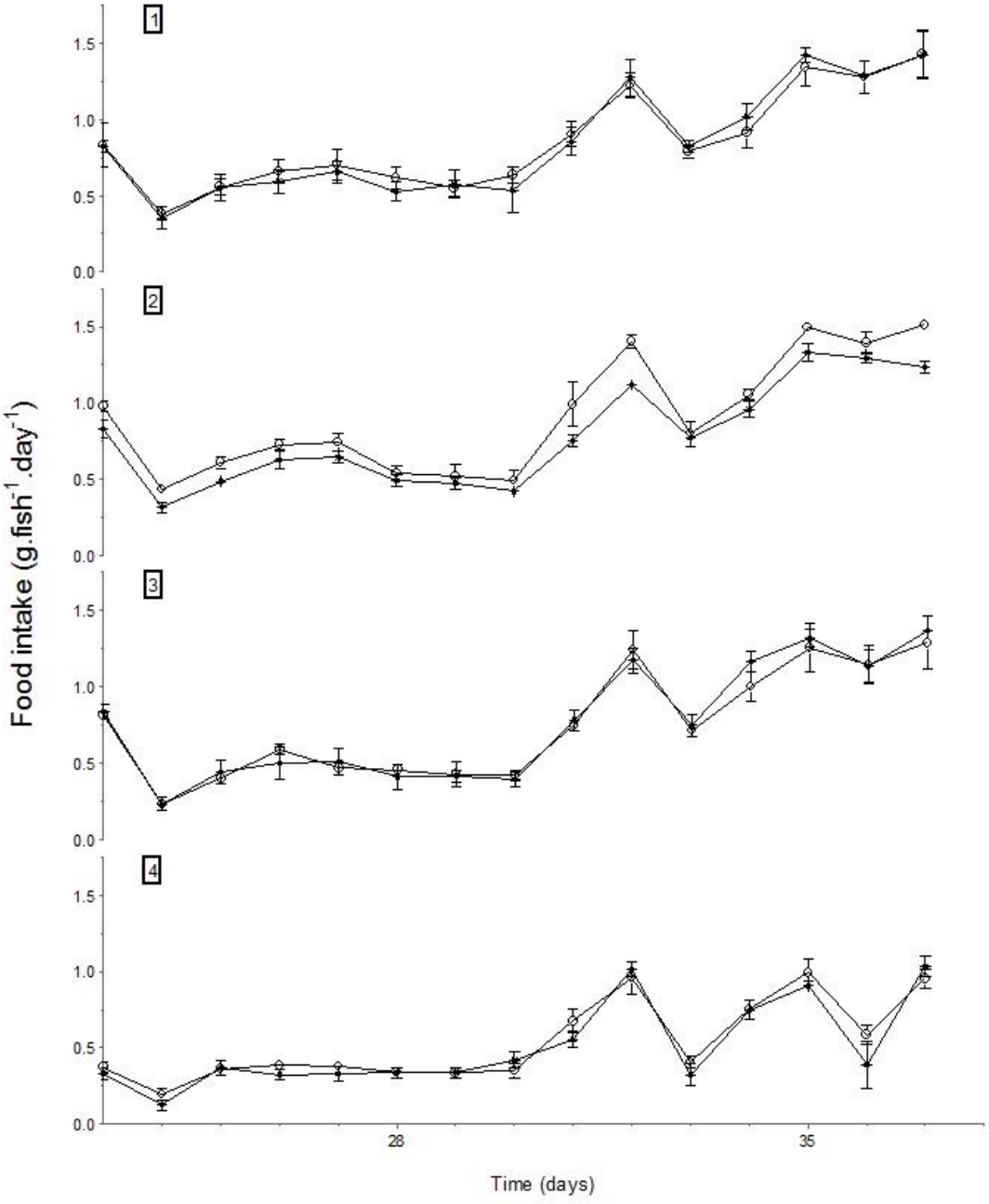

Figure 4.2 Food intake (g.fish^{-1}.day^{-}1) post saltwater transfer (phase 4) of fish fed modified feeds containing TRP at: Feed 1, 4.9 mg.g^{-1}; Feed 2, 10.9 mg.g^{-1}; Feed 3, 21.8 mg.g^{-1}; and Feed 4, 46.3 mg.g^{-1} when compared to either low stress (○) or high stress (⁎) transfer technique. Data are mean values ±SEM. Fish consuming a feed containing 10.9 mg.g^{-1} (**2**) and subjected to low stress transfer fed more strongly (t = 6.22, *df* = 14, p < 0.0001) than fish on the same feed but subjected to high stress transfer when compared with a paired t-test. No other levels of dietary TRP elicited a change in food intake post saltwater transfer.

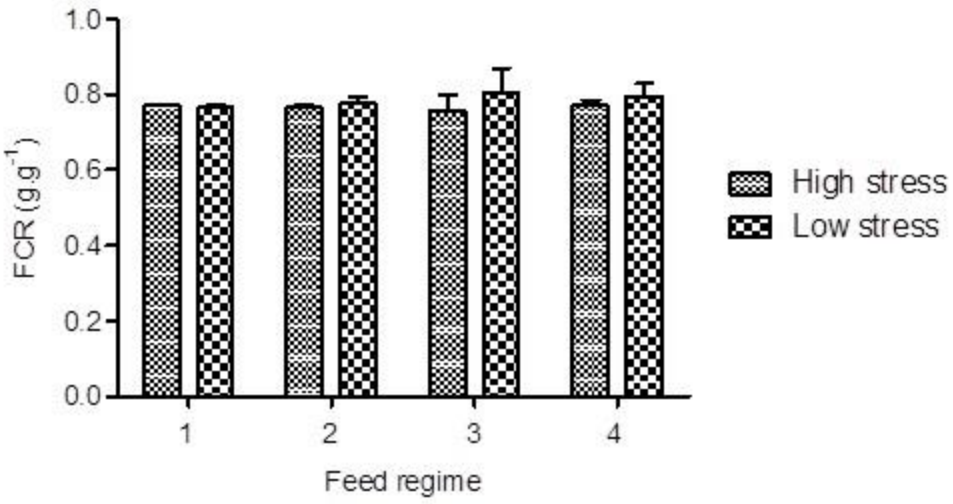

Figure 4.3 Feed conversion ratio (FCR) for each dietary (Feed 1, 4.9 mg.g^{-1}; Feed 2, 10.9 mg.g^{-1}; Feed 3, 21.8 mg.g^{-1}; and Feed 4, 46.3 mg.g^{-1})and saltwater transfer treatment (mean ±SEM) over the duration of the experiment. No differences between treatments were observed.

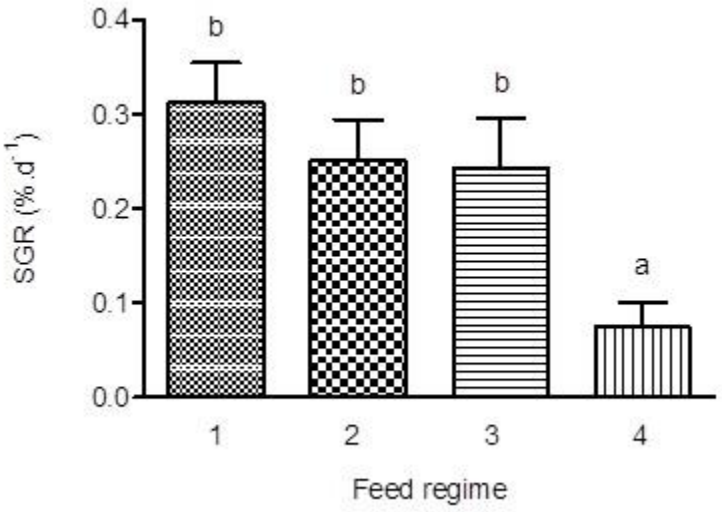

Figure 4.4 Specific growth rate (%.d^{-1}) of Atlantic salmon smolts fed 1 of four feeds containing TRp at**:** Feed 1, 4.9 mg.g^{-1}; Feed 2, 10.9 mg.g^{-1}; Feed 3, 21.8 mg.g^{-1}; and Feed 4, 46.3 mg.g^{-1}, and presented as mean ± SEM throughout a 45 day period over saltwater transfer. ANOVA showed a difference between treatments (F 5.86, *df* 3, 20, $p < 0.005$). Different superscript letters denote differences between treatment means (Tukey's multiple comparison test $p < 0.05$).

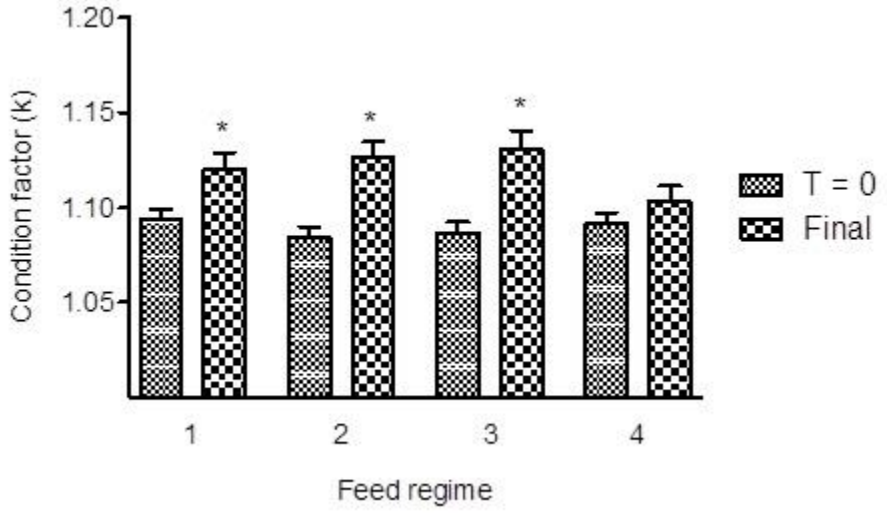

Figure 4.5 Condition factor (k) of fish offered feeds (mean ± SEM) containing different quantities of TRP at Feed 1, 4.9 $mg.g^{-1}$; Feed 2, 10.9 $mg.g^{-1}$; Feed 3, 21.8 $mg.g^{-1}$; and Feed 4, 46.3 $mg.g^{-1}$, at the beginning and at the end of the experiment with asterisks denoting a difference with the previous column. All dietary treatments observed increases in condition factor (1, $t=2.79$ $df=309$, $p < 0.05$; 2, $t=4.53$ $df=303$, $p < 0.0001$; 3, $t=4.20$ $df=309$, $p < 0.0001$, with the exception of feed 4.

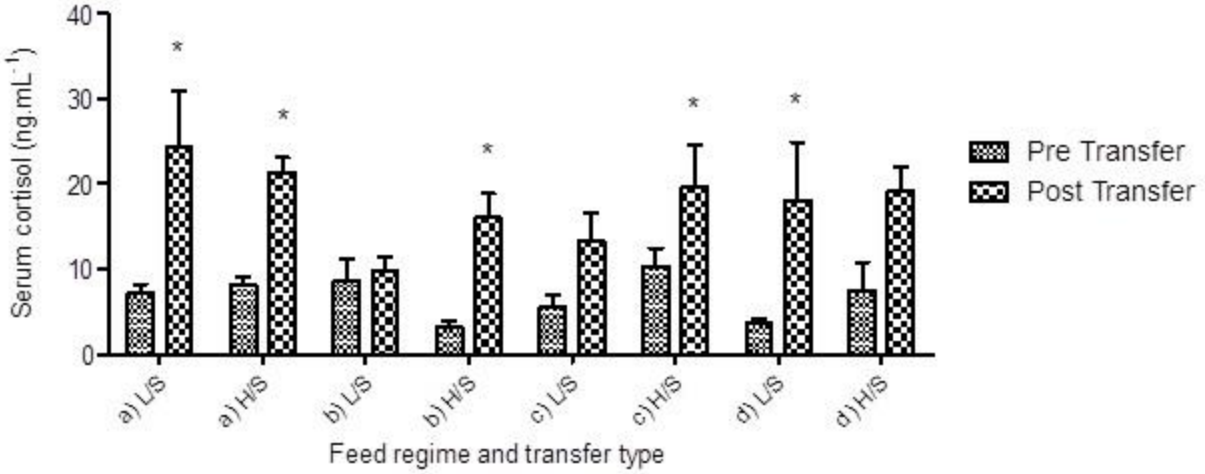

Figure 4.6 Serum cortisol (ng.mL^{-1}) concentrations (mean ± SEM)of Atlantic salmon smolts pre and post (48h) saltwater (30ppt) transfer when fed feeds containing TRP at **a**) Feed 1, 4.9 mg.g^{-1}; **b**) Feed 2, 10.9 mg.g^{-1}; **c**) Feed 3, 21.8 mg.g^{-1}; and **d**) Feed 4, 46.3 mg.g^{-1}. Two way ANOVA showed significant difference (F 71.0, *df* 1, 170, $p < 0.0001$) between pre-transfer and post-transfer serum cortisol. No differences were observed for transfer type. L/S indicates low stress transfer, H/S indicates high stress transfer. Asterisks denote a difference (Bonferroni $p < 0.05$) with the preceding column.

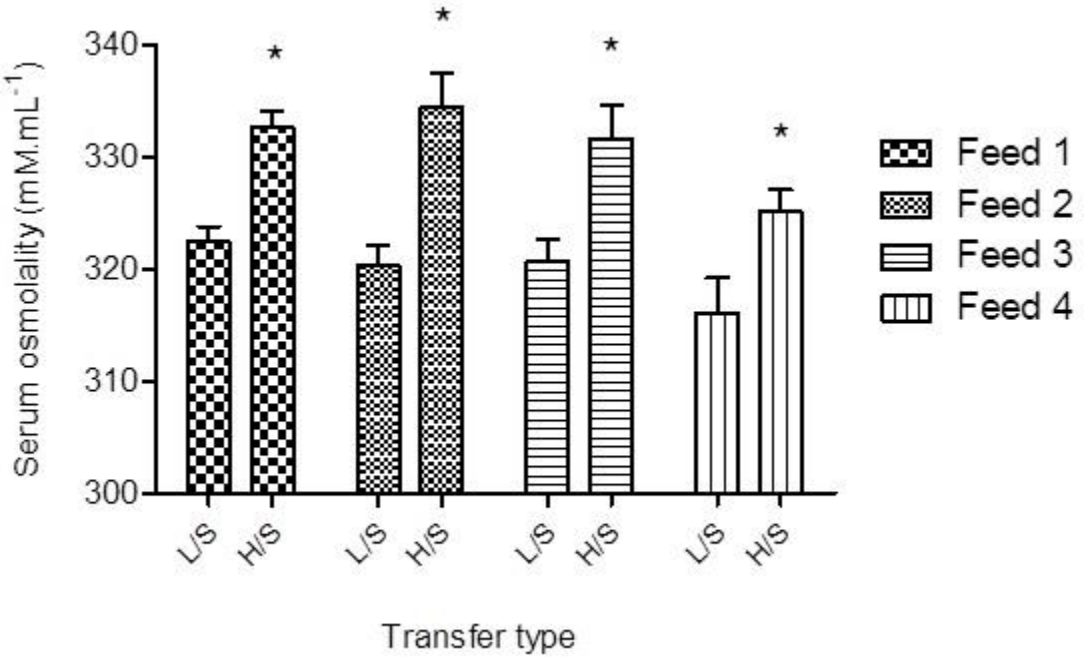

Figure 4.7 Serum osmolality ($mM.mL^{-1}$) of Atlantic salmon smolts post (48hrs) saltwater (30ppt) transfer relative to either high stress or low stress transfer method when fed feeds containing TRP at: Feed 1, 4.9 $mg.g^{-1}$; Feed 2, 10.9 $mg.g^{-1}$; Feed 3, 21.8 $mg.g^{-1}$; and Feed 4, 46.3 $mg.g^{-1}$. Data are displayed as mean ± SEM. Two way ANOVA of feed and transfer type showed significant differences ($F = 3.99$, *df* 3, 104, $p < 0.05$; $F = 44.35$, *df* 1, 104, $p < 0.0001$) respectively. Asterisks denote a difference (Bonferroni $p < 0.05$) with the preceding column.

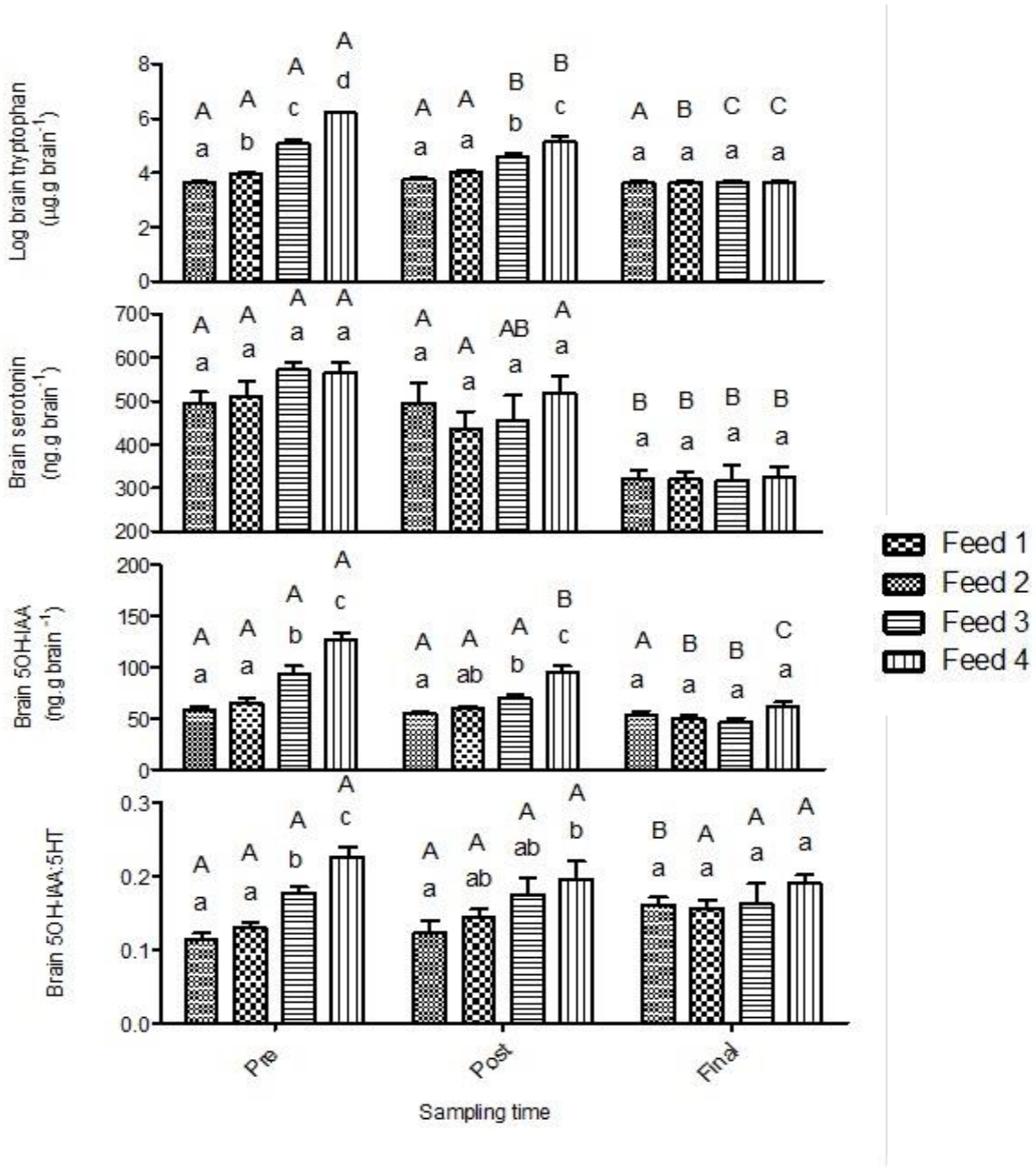

Figure 4.8 Tryptophan and associated neurotransmitters extracted from whole brains of Atlantic salmon smolts fed pelleted feed containing varying levels of supplementary TRP pre saltwater transfer (Pre; 0 ppt), post saltwater transfer (Post; 30 ppt), and after resuming commercial feed for 8 days (Final; 30 ppt). Mean data were log transformed for analysis and presented as non-transformed data, except for brain tryptophan which remains log transformed for clarity. Differences between feeds within sampling times are indicated by different lowercase superscript letters (Tukey's multiple comparison test $p < 0.05$). Differences within feeds across sampling times are indicated by different uppercase superscript letters (Tukey's multiple comparison test $p < 0.05$).

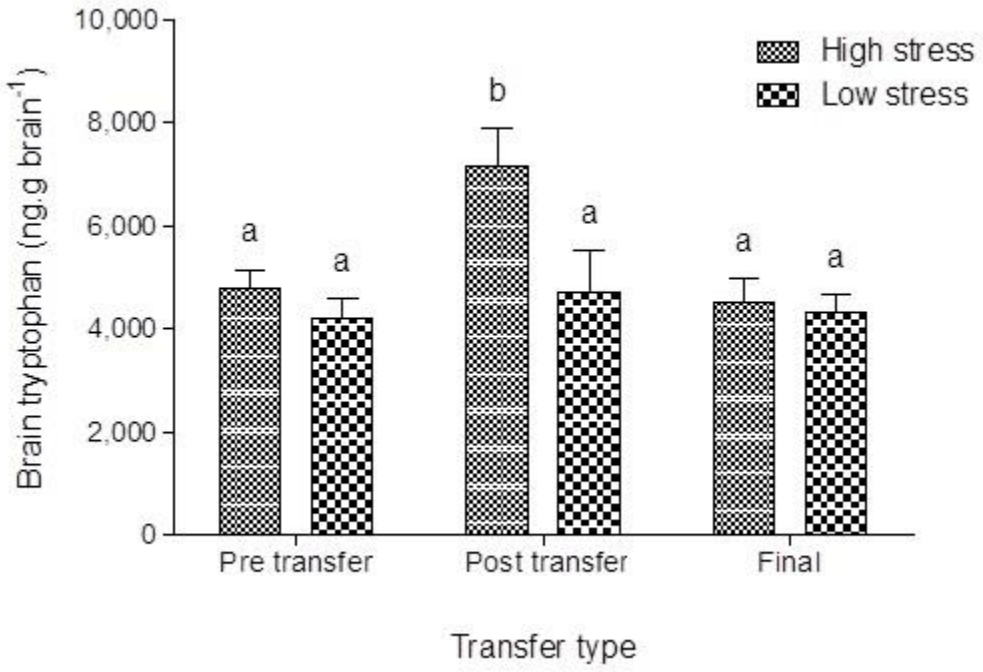

Figure 4.9 Brain tryptophan (ng.g.brain^{-1}) concentrations (mean ± SEM) of Atlantic salmon smolts pre and post (48h) saltwater (30ppt) transfer, and at the culmination of the experiment, for fish fed the reference feed (Feed 1, 4.9 mg.g^{-1}). Two way ANOVA showed significant difference (F 4.25, *df* 2, 26, $p < 0.05$) between at post-transfer sampling between high stress and low stress transfer type. Asterisks denote a difference (Bonferroni $p < 0.05$) between columns.

Table 4.4 Performance of Atlantic salmon smolts fed pelleted feed containing varying levels of supplementary TRP throughout a 45 day period over saltwater transfer. Difference between growth performance means are identified by differing letters (Tukey's multiple comparison test, $p < 0.05$). No differences were observed between treatments for survival (Kaplan Meier).

	Feed 1	Feed 2	Feed 3	Feed 4	F	*df*	p
Weight (g)							
Initial	86.1 ± 0.735 N=186	85.9 ± 0.875 N=184	87.4 ± 0.853 N=186	88.2 ± 0.870 N=184	1.72	3, 736	0.161
Day 21 weight	118 ± 2.64 N=30	115 ± 3.29 N=30	123 ± 3.06 N=30	112 ± 3.07 N=30	2.32	3, 116	0.079
Day 23 weight	112 ± 3.28 N=30	117 ± 3.11 N=30	116 ± 3.11 N=30	111 ± 2.75 N=30	1.06	3, 116	0.370
Pooled Day 21 & Day 23	115 ± 2.12 N=60	116 ± 2.25 N=60	119 ± 2.21 N=60	111 ± 2.04 N=60	2.42	3, 236	0.066
Final weight	148 ± 2.27 N=125 (a)	146 ± 2.36 N=121 (ab)	146 ± 2.54 N=124 (ab)	139 ± 2.02 N=121 (b)	3.13	3, 487	0.025*
Weight gain (tank)	1567 ± 63.4 N=6 (a)	1522 ± 29.0 N=6 (a)	1526 ± 46.7 N=6 (a)	1236 ± 26.0 N=6 (b)	12.1	3, 20	< 0.001*
Length (mm)							
Initial	199 ± 0.601 N=186	199 ± 0.677 N=184	200 ± 0.631 N=186	200 ± 0.689 N=184	1.45	3, 736	0.226
Day 21 length	213 ± 1.47 N=30	212 ± 2.12 N=30	216 ± 1.61 N=30	211 ± 1.81 N=30	1.65	3, 116	0.182
Day 23 length	211 ± 2.17 N=30	216 ± 2.39 N=30	216 ± 2.28 N=30	214 ± 1.86 N=30	1.01	3, 116	0.392
Pooled Day 21 & Day 23	212 ± 1.30 N=60	214 ± 1.61 N=60	216 ± 1.38 N=60	212 ± 1.30 N=60	1.48	3, 236	0.220
Final length	236 ± 1.26 N=125	234 ± 1.33 N=121	234 ± 1.44 N=124	232 ± 1.22 N=121	1.33	3, 487	0.265
Change in length (tank)	36.9 ± 0.906 N=6 (a)	35.5 ± 1.45 N=6 (a)	33.7 ± 1.05 N=6 (a)	31.5 ± 0.831 N=6 (b)	4.53	3, 20	0.014*
Survival (%)	99.5	98.4	99.5	98.9	x^2 1.625	3	0.653

4.4 Discussion

This study showed that the addition of supplementary dietary TRP did not ameliorate feed intake depression typically observed post-SWT nor did it decrease the time for which a reduction in feed intake was observed, however extreme inclusion (46.3 $mg.g^{-1}$; feed 4) of TRP in the diet was shown to reduce feed intake both pre and post-SWT. Fish consuming a feed containing 10.9 $mg.g^{-1}$ (feed 2) and subjected to low stress transfer fed more strongly than fish on the same feed but subjected to high stress. No other levels of dietary TRP elicited a change in feed intake post saltwater transfer relative to SWT method. Serum cortisol was found to be higher post-SWT compared to pre-SWT however supplementary dietary TRP was not found to affect circulating cortisol. Serum osmolarity was greater post-SWT for fish subjected to the high stress transfer.

There were a number of unavoidable issues with the experiment including the occurrence of skeletal and jaw deformities, and the all-female triploid nature of the fish. Furthermore, it is acknowledged that the feeding regime, at twice daily to satiation, did not mirror current industry practice. Sourcing of the fish was subject to availability (as mentioned in the Materials and Methods section) as the University of Tasmania does not currently have the scope to produce smolt for research purposes. Lower jaw deformity is common in cultured triploid Atlantic salmon in Tasmania and has been shown to occur in up to 30% of the population (Lijalad and Powell 2009). Differences in growth and feeding behaviour have been exhibited between triploid and diploid Atlantic salmon parr (Carter *et al.* 1994). In seawater triploids have improved growth performance over diploids, however also exhibit increased occurrence of jaw deformities and reduced survival (O'Flynn *et al.* 1997). Fish with lower jaw and skeletal deformities were excleuded from the experiment. However, it is acknowledged that the results and conclusions from the presented data may not be directly applicable to diploid stock.

4.4.1 Food intake and growth

In the present study it was hypothesized that the severity of the reduction in the feed intake of Atlantic salmon smolts at SWT may be associated with stressors associated with transfer, such as crowding (Schreck CB *et al.* 1997), other than those associated with change in chemical

composition of the aquatic environment (Damsgard and Arnesen 1998) and that supplementary dietary TRP, the precursor of 5HT, a neurotransmitter associated with mood modulation in mammals, may alleviate the corticosteroid response in Atlantic salmon at SWT with a consequent positive effect on feed intake. Due to the lack of appropriate fish weight data throughout the experiment and the consequent inability to deliver an accurate measure of feed consumption relative to bodyweight, feed intake is described in grams per fish per day (Flood *et al.* 2011).

The transfer to a 30 ‰ seawater environment was conducted over 48 hours with the initial change on day 21 being from FW to 20 ‰ and a steady increase to 30 ‰ over the ensuing time. Food intake responses for fish on all feeds were similar with a strong correlation between increasing salinity and reduced food intake presumably as a result of the physiological adaptations to higher salinities and the increase in drinking of seawater to compensate for the loss of water via osmosis to the hyperosmotic environment. In this study the severe depression in food intake across all feeds lasted 10 days after initial exposure to higher salinity water, concurrent with other work showing an increase in rates of drinking from 0 $mL.Kg^{-1}h^{-1}$ in FW to 6.9 $mL.Kg^{-1}h^{-1}$ over 9 days (Usher *et al.* 1988). Though Usher et al. (1988) observed in Atlantic salmon smolts that "under conditions of normal, volitional feeding, distension of the gut was neither sufficiently severe nor sustained long enough to interrupt drinking" earlier studies on *Anguilla japonica* (Hirano 1974) and *Gadhus morhua* (Holstein 1979) showed that distension of the stomach and intestine depressed further water intake, with the suggestion posited that volume sensitive receptors neurally linked with the CNS were the trigger (Holstein 1979). Some of the reduction in food intake by Atlantic salmon smolts post-SWT may be caused by the sensation of fullness through stomach distension as a result of increased imbibing of seawater, as it is generally accepted that fish stomach distension has an inhibitory effect on appetite (De Silva 1995).

A lack of difference in food intake between treatments and an apparent increase in consumption over time during phase 1 indicates that there was no underlying variability prior to the introduction of experimental feeds. The continuation of this trajectory during phase two

for all feeds except feed 4 suggests the inhibitory effect of 5-HT on appetite is not apparent in Atlantic salmon smolts until dietary inclusion exceeds 21.8 $mg.g^{-1}$. The feed response on day 14, the first day of phase 2 and the introduction of experimental feeds, to feed 4 was not reduced from food intake during phase 1 indicating that palatability was probably not a factor in reduced food intake later in the phase. A high inclusion of TRP in the diet (46.3 $mg.g^{-}1$; feed 4) was found to inhibit food intake presumably as a result of elevated brain serotonin, a condition known to deliver a strong hypophagic response in rodents and humans (Leibowitz and Alexander) (Blundell and Halford 1998; Halford *et al.* 2007) as well as in teleost fish (De Pedro *et al.* 1998a; Lin *et al.* 2000; Ortega *et al.* 2013). This effect is mediated via 5-HT receptors located in various medial hypothalamic nuclei (Leibowitz and Alexander 1998). Pre-treatment with the corticotropin-releasing factor (CRF) antagonist α-CRF_{9-41} partially blocked the hypophagia in goldfish, suggesting CRF may have a role in mediating a 5-HT induced reduction in food intake (De Pedro *et al.* 1998a). A further study on rainbow trout showed an elevation in telencephalic levels of 5OH-IAA and 5OH-IAA : 5HT following long term food deprivation further suggesting the role of the serotonergic system in appetite mediation and intake control (Ruibal *et al.* 2002). Behavioural inhibition in various teleost species, including suppression of feeding, in subordinate animals results in a chronic up regulation of the serotonergic system, whereas this affect appears to be mediated in dominant fish by dopamine (Winberg and Nilsson 1993a, b; Overli *et al.* 1999). No correlation between increased dietary TRP and serum cortisol, produced in the head kidney in response to CRF from the hypothalamic nucleus preoptic, was observed in the current study and therefore mediation of hypophagia via elevated TRP was not expected to have occurred. Furthermore, correlations between serum cortisol and 5HT or 5HIAA were also not observed. No correlation was observed between individual size (length) and any of the circulating or neuroendocrine measures recorded, however the experiment did not examine or record individual position within feeding or social hierarchies.

The brevity of phase 2 may occlude a potential return of feed 4 intake to one in line with the other feeds, however the steady increase in food intake for fish fed feed 4 over days 16 to 20 inclusive was disrupted by SWT at day 21 which had a very strong negative effect on food

intake for all feeds, a finding consistent with other studies (Usher *et al.* 1991; Stead *et al.* 1996; Flood *et al.* 2011). Food intake was significantly greater immediately post-SWT for fish fed feed 2 which were subjected to a low stress transfer than for fish on the same feed but subjected to a high stress transfer, however analysis of the food intake of these two groups during phase 1 showed a significant difference for that period also and thus the difference exhibited post-transfer is considered to reflect a continuation of the observed pre-transfer feeding, although at a reduced rate as a result of transfer, rather than a moderation of feeding depression attributable to the inclusion of supplementary TRP in the feed. Furthermore the difference is apparent during days 1-13, which is prior to the administration of feeds with supplementary TRP. No other differences in food intake were detected for transfer type.

A lack of difference in FCR between feeds suggests that supplementary TRP in levels up to 46.3 $mg.g^{-1}$ has no adverse effect on nutrient digestibility and adsorption, and that the depressed growth performance , as observed by reduced SGR and final weights of fish fed feed 4, can be attributed to the reduction in intake. It is acknowledged that the calculations for both FCR and SGR are calculated over the entire course of the experiment and that differences relative to TRP content may have been observed if each feed phase were longer and growth performance were presented by phase, however the associated requirement for additional fish handling during the experiment may have had a detrimental effect on a number of the parameters studied. The increase in condition factor (k) over the course of the experiment for fish fed all feeds except feed 4 possibly reflects the poor condition of fish on arrival in conjunction with reduced food intake of feed 4 during phases 2 through 4 inclusive.

4.4.2 Osmolality and endocrine response

The effect of SWT type was consistent across all feed treatments with an elevation in serum osmolality associated with high stress transfer (HST). This effect is thought to be most likely attributable to the initially higher salinity that the HST fish were subjected to as those tanks were almost completely drained and then filled directly with 30 ‰ seawater. The salinity was

then unintentionally reduced to 20 ‰ as a result of resuming the flow of the recirculating system, while the introduced granulated salt sufficient to bring the total system volume to 30 ‰ remained undissolved overnight, before returning to 30 ‰ 24 h after the initial transfer. Osmolality was conducted at 48 h post-SWT which was 24 h after system salinity reached 30 ‰.

Transfer type was not found to have an effect on serum cortisol however overall the effect of transfer did with higher responses from post-transfer sampling compared to pre-transfer sampling. Fish were sampled in the same manner both pre and post-transfer, so assuming that no effects derived from unknown differences in sampling time / technique between days are apparent then it can be concluded that the transfer to a 30 ‰ environment from a 0 ‰ environment delivers a stress response in Atlantic salmon smolts that remains evident 48 h after initial change in salinity. This response in salmonids has been observed elsewhere (Young *et al.* 1989; Franklin *et al.* 1992)

Serum cortisol concentrations pre and post-SWT, for the low stress SWT group of fish fed feed 2 are the most similar; indeed the serum cortisol response for this group is the lowest recorded post-transfer. Though higher levels of circulating cortisol, naturally observed in association with smoltification and SWT (Specker and Schreck 1982; Franklin *et al.* 1992; Mommsen *et al.* 1999), are widely thought to inhibit food intake in teleost fish there is a scarcity of literature in this area. A loss of appetite and aggressive feeding behaviour was reported in rainbow trout administered a feed containing supplementary cortisol (Barton *et al.* 1987a) however all feed presented was eaten. Conversely, a more recent study on the effects of dietary cortisol on goldfish found a dose dependent orexigenic effect on food intake for fish with plasma cortisol concentrations of approximately 50 $ng.mL^{-1}$ (Bernier *et al.* 2004). Reduced food intake was observed at high (approximately 275 $ng.mL^{-1}$) concentrations. In the current study serum cortisol (ca. 3 – 30 $ng.mL^{-1}$) was not observed at levels which stimulated food intake in goldfish (ca 50$ng.mL^{-1}$) and neither was it at the level which inhibited feeding (circa 275 $ng.mL^{-1}$) (Bernier *et al.* 2004). However the circulating cortisol response to SWT would most likely have stabilized by the time post-transfer samples were taken, for example sockeye salmon (*Oncorhynchus nerka*) have a peak plasma cortisol response of circa 240 $ng.mL^{-1}$ at 1 to 2 h

post-transfer which stabilizes to within the range seen in this experiment after 24 h (Franklin *et al.* 1992). Though no correlation was observed between supplementary dietary TRP and serum cortisol, either before SWT or 48h post-SWT, pre-SWT results conform with reported data for unstressed Atlantic salmon smolts (Pankhurst *et al.* 2008) suggesting that supplementary dietary TRP does not elevate serum cortisol in unstressed Atlantic salmon smolts at any of the inclusions tested. This appears to be contrary to previous salmonid research which concluded that 5HT stimulated activity of the HPI axis in rainbow trout (Winberg *et al.* 1997). The subsequent positive feedback loop is also observed in other species, as introcerebroventricularly administered CRF activates TRP hydroxylase, the rate limiting step of the 5HT biosynthesis pathway (De Simoni *et al.* 1987), in rats (Dunn and Berridge 1990), and lower ranking Arctic charr (*Salvelinus* alpinus) exhibit elevated serotonergic activity attributed to higher circulating glucocorticoids (Winberg *et al.* 1992; Winberg *et al.* 1993). The current study however doesn't report any differences between feed treatments for brain 5HT. There remains the possibility that supplementary dietary TRP may mitigate a cortisol response in Atlantic salmon smolts at SWT, as in this experiment sampling was not conducted at times to coincide with peak response, however the similar food intake between feeds (except for the high inclusion feed 4) suggests that no such action occurred or if it did it was insufficient to affect feeding behaviour. It is of course possible that elevated circulating cortisol, in the context of food intake, should be viewed primarily as a useful physiological predictor of feeding suppression (Pankhurst *et al.* 2008), though does not in itself induce hypophagia as suggested by De Pedro *et al.* (De Pedro *et al.* 1997). Maybe CRF, ACTH or other HPI associated factors, hormones and receptors play a more dominant role, or possibly the concomitant up-regulation of the serotonergic system is a more likely trigger.

The actions of the HPI axis stress response in fish have were described in the middle of the 20th century, however knowledge of the exact pathways of behavioural modulation, such as feeding inhibition remain incomplete. Corticotropin releasing factor, a 41-amino acid bioactive peptide derived from a 160 amino acid preprohormone first described in 1981 (Vale *et al.* 1981) is strongly implicated. CRF is part of a family of peptides which in teleost fish also includes three urotensins UTn1, UTn2 and UTn3, and is found in all vertebrates. The stress response in fish is

characterized by hypothalamic CRF release which stimulates pituitary adrenocorticotropic hormone (ACTH) in the pituitary gland via neurosecretory fibres, and subsequent glucocorticoid synthesis and release from the interrenal tissue. CRF synthesis is primarily via peptidergic neurons in the nucleus preoptic (NPO) of the hypothalamus, however is also expressed in extrahypothalamic brain regions and in peripheral tissues such as the caudal neurosecretory system (Flik G *et al.* 2006). The eventual CRF signal is determined by the CRF structure, the presence of CRF binding protein (CRF-BP), and the CRF receptor (CRF-R1 or CRF_R2). All of the CRF-like peptides are thought to bind and consequently their function may be modified by CRF-BP (Ronan and Summers 2011). The effects of CRF have been shown to be mediated by receptor type with CRF-R1 reported to mediate the activation of the HPI axis (Huising *et al.* 2004) and to induce anxiety like behaviour in mice (Heinrichs *et al.* 1997). CRF-R2 have been implicated in anxiety control in mice (Bale *et al.* 2000) as well as a number of other behavioral and physiological adaptations to stress response such as prolonged hypophagia and associated weight loss (Coste *et al.* 2006). Furthermore ICV administration of peptides from the CRF group have been shown to inhibit food intake in a dose dependent manner in goldfish (De Pedro *et al.* 1993; Bernier and Peter 2001a), and feeding suppression in hypoxic rainbow trout has also been partially attributed to increased CRF-related peptide activity (Bernier and Craig 2005). More recent research on cortisol-administered, chronically stressed rainbow trout also implicates NPO CRF as well as liver leptin, a hormone associated with satiety, as mediators of reduced food intake (Madison *et al.* 2015). More tellingly, in rainbow trout subjected to SWT, feeding suppression coincided with up regulation of hypothalamic CRF and UI mRNA, and chronic elevation of caudal neurosecretory system (CNSS) CRF and UI gene expression, suggesting that CRF related peptides may serve as regulators of food intake at SWT in salmonids (Craig *et al.* 2005).

Unsurprisingly, in such an evolutionarily conserved system with numerous outcomes, CRF synthesis is not only stress related. Studies on rats have shown that 5HT stimulates CRF release, which interacts with 5HT receptors on CRF neurons in the paraventricular nucleus PVN (homolog to the NPO in fish), and activates CRF synthesis both in vitro (Nakagami *et al.* 1986; Itoi *et al.* 1998) and in vivo (Kageyama *et al.* 1998). Studies on teleost fish are more limited,

however Medeiros *et al.* (2014) demonstrated an increase in hypothalamic CRF precursor DNA and subsequent pituitary ACTH release in toadfish (*Opsanus beta*) after intravenous injection of the $5HT_{1A}$ receptor agonist, 8-OH-DPAT. The same authors also present evidence that chronically elevated plasma cortisol attenuates the $5HT_{1A}$ receptor mediated secretion of both CRF precursor and ACTH.

The control of food intake in fish involves a complex and not well understood interaction of numerous direct and indirect responses, many of which are subject to feedback loops. Tempting though it is, this thesis is not the place for an exhaustive review of food intake in fish. In addressing one of the aims of this study, namely to assess whether supplementary dietary TRP moderates the feeding depression observed in Atlantic salmon smolts at SWT, it has been pertinent to assess which of the feeding inhibitory inputs may have been implicated in the current results. Though not dismissing other axes it seems that elevated NPO-derived and possibly CNSS-derived CRH, maybe as a consequence of salinity stress and SWT type, but predominately from elevated dietary TRP suppressed food intake in a dose dependent manner in Atlantic salmon smolts at salt water transfer.

4.4.3 Neuroendocrine response

The differences observed in brain TRP pre-SWT reflect the inclusion of TRP in the feed combined with the rate of ingestion. The lack of difference between brain TRP concentrations of fish fed feed 1 and feed 2, and the reduction in values for feeds 3 and 4 post-SWT are probably as a result of the reduction in food intake associated with SWT. No differences were detected between brain TRP concentrations recorded at final sampling, which all conformed with prior sampling from feed 1 animals. This suggests that waterborne TRP contamination was not a factor, and that despite previously highly elevated concentrations of brain TRP, all fish sampled were returned to expected basal concentrations within 7 days of resuming a commercial feed. The increase in brain [TRP] following SWT in fish subjected to high stress transfer compared to those subjected to low stress transfer is in accordance with other findings

showing that stress increases brain [TRP] (Winberg and Nilsson 1993a). Interestingly this response is only present in fish subjected to the high stress transfer however an elevated serum cortisol response was evident at this stage for both high and low stress transfer. This might suggest a different mechanism is involved in the responses to SWT and crowding. Both produce a chronic cortisol response, however only the high stress transfer produces prolonged elevation of brain [TRP].

No differences in brain serotonin were observed at any of the sampling times between feeds suggesting either an inability of Atlantic salmon smolts to metabolise brain TRP into 5HT at levels much beyond those commonly encountered, or a delayed increase in serotonergic capacity. The relative stability of brain 5HT in salmonids has been observed elsewhere (Winberg and Nilsson 1993b). The current study appears to confirm the idea that, though TRP is the precursor of 5HT, elevation of circulating and brain TRP concentrations seem to increase intraneuronal 5HT catabolism without an attendant 5HT release (Lookingland K *et al.* 1986; De Simoni *et al.* 1987). Brain 5HIAA rather than 5HT therefore provides a much clearer picture of both the 5HT stress response and the serotonergic response to elevated brain TRP, as observed by a lack of correlation between brain TRP and brain 5HT in the current study. The strong positive correlations between 5HIAA and both TRP and 5HT describe much more clearly the up-regulation of the serotonergic system at pre and post-SWT sampling as a result of elevated dietary TRP. Interestingly at final sampling in the present study negative correlations existed for growth performance measures (length and weight) with 5HT and 5HIAA indicating that the effect of elevated dietary TRP at earlier time-points may have occluded the stress related 5HT response in smaller, probably subordinate fish. This has been noted elsewhere in response to social hierarchies (Cubitt *et al.* 2008), however size variation of fish in the current study was intentionally limited and thus a size related response may have been masked at earlier time-points when the coefficient of variation (CV) of growth performance was much more limited. This observation is supported by weak correlations for both length and 5HIAA, and TRP and 5HT when data were pooled from pre and post-SWT of fish fed feed 1. Furthermore it appears that a more specific study of non TRP supplemented diets and cortisol response in Atlantic salmon at SWT may yield a clearer picture of the relationship between the HPI stress response and

neuroendocrine stimulation of the serotonergic system, as data from this study suggest a direct link may be evident under these circumstances. This would back up numerous studies that have confirmed manipulation of the serotonergic system to have consequent effects on the HPI and HPA axes (Winberg *et al.* 1997).

The significant reduction in brain serotonin concentrations at final sampling across all feeds is difficult to explain. Though a reduction would be expected across feeds 2, 3 and 4 in line with reduced prevalence of brain TRP, the expectation would be for a more uniform response between sampling times for feed 1. There is no reason to believe sampling method or time, tissue processing or machine error may have confounded these data, and therefore an assumption has been made of brain 5HT concentrations at final sampling as being most representative of normal levels. The relative elevation of brain 5HT concentrations for feeds 2, 3 and 4 at pre and post-SWT sampling times can be at least partially attributed to supplementary dietary TRP. The stimulation of serotonergic activity as a consequence of chronic stress may explain the apparently elevated concentrations of brain 5HT at pre and post-SWT sampling however there is no attendant elevation of cortisol. Acute stressors administered to rainbow trout for as little as 15 seconds have been shown to immediately elevate brain 5HT:5HIAA and for the effect to remain for 4 hours (Gesto *et al.* 2013). Smoltification in salmonids, a process associated with increased plasma cortisol levels (Specker and Schreck 1982; Mommsen *et al.* 1999), triggered by age of fish and environmental factors such as water temperature and photoperiod manipulation, and known in this instance to have occurred from osmolality data, is a plausible causal factor in elevated serotonergic activity observed at pre-SWT sampling. It is also possible that the cortisol spike associated with SWT, subdued by the time of post-SWT sampling, may have also triggered an up regulation of serotonergic activity. Furthermore during chronic stress, plasma cortisol levels have been observed to return to resting levels despite continued response to the stressor (Vijayan and Leatherland 1990).

Given that, as previously mentioned, the level of brain 5HT appears to be relatively stable irrespective of TRP input, the ratio of 5HIAA : 5HT, with 5HIAA being the more variable factor, is commonly used to describe the activity of the 5HT system. In the current study 5HIAA : 5HT

reflects dietary TRP levels at pre and post-SWT however at final sampling there is no reduction, in fact a slight increase is evident across feeds 1 and 2. It is not inconceivable that a continued serotonergic response could be evident at final sampling 24 days post-SWT and 7 days post cessation of experimental feeds, associated with the HPI response to either the elevated dietary TRP, though this seems unlikely given the response of the reference feed, or with the HPI response associated with SWT itself. Subordinate Arctic charr showed continued elevated 5HIAA : 5HT in the telencephalon, hypothalamus and brain stem up to 21 days post introduction of another same sized conspecific at a time when brain TRP and 5HT remained at basal (control) levels, attributed to an up regulation of the 5HT neurotransmitter systems presumably in response to HPI activity (Winberg and Nilsson 1993b). It remains possible that the lack of clear reduction in 5HIAA : 5HT at final sampling is a reflection of a processing error of the 5HT samples, which delivered unexpectedly low values.

Saltwater transfer elicits a serum cortisol response in Atlantic salmon smolts 48 h after transfer however SWT type appeared not to have an effect at this stage. There is a suggestion of confirmation of a direct link between the HPI axis and the serotonergic system. Serum osmolarity is affected by transfer type with a high stress transfer eliciting a stronger response, which is presumed to reflect the earlier exposure to 30 ‰ water. Both the higher and lower responses fall within a standard expected range and, though of interest are not thought to be biologically important. An elevation in serotonergic activity in response to supplementary dietary TRP is evident despite relatively static 5HT levels and is thought to reflect an up regulation of the 5HT neurotransmitter systems possibly via stimulation of the dorsal raphe.

In conclusion there is no advantage in feeding Atlantic salmon smolts feeds supplemented with dietary TRP in the range of 10.9 $mg.g^{-1}$ to 46.3 $mg.g^{-1}$ in an effort to reduce the impact of SWT on food intake, conversely, at 46.3 $mg.g^{-1}$ food intake was greatly reduced. Therefore the hypothesis: Supplementing fish food with TRP will increase food intake in Atlantic salmon smolt at SWT compared to those fed a non-TRP supplemented feed, can be rejected.

5 Chapter 5 - General Discussion

5.1 Overview of study

The current study focused on the use of supplementary dietary TRP for two species, barramundi and Atlantic salmon, of commercial importance in Australia. Experiments were designed so that changes in behaviour, rates of cannibalism, food intake, growth performance, physiological stress response and serotonergic activity could be evaluated relative to dietary inclusion of TRP, during a period of juvenile development and, in the case of Atlantic salmon, transfer from fresh water to sea water.

Aggressive interactions between fish contribute significantly to the development of hierarchies, and the suppression of feeding and subsequent poor growth performance in subordinate individuals. Subordination in itself is a significant stressor and one which triggers both a corticosteroid and serotonergic response. Both of these responses are associated with reduced food intake. Reduced food intake results in growth depensation, which not only favours the dominant individual, but has significant commercial drawbacks. Fierceness, and the result of previous interactions, are key determinants of hierarchical structure.

Tryptophan is the precursor amino acid of serotonin, a monoamine neurotransmitter, with a complex array of functions. Some of these functions, such as the modulation of aggressive behaviours in aggressive individuals and the increase in confrontation of subordinate individuals, the moderation of corticosteroid response in stressed individuals, and the anorectic effect, make it appear to be a suitable supplementary ingredient for the culture of aggressive fish species at high densities such as those used in commercial production.

Supplementary dietary TRP elicited in this study a hypophagic response in juvenile barramundi and Atlantic salmon smolts, with the severity of the effect related to dietary inclusion, food consumption, and fish size. The growth (SGR) of juvenile barramundi was negatively impacted by supplementary TRP via both hypophagia and a poorer FCR. Brain [TRP] in juvenile barramundi appeared to be affected by ration size, suggesting large neutral amino acid (LNAA) competition at the blood brain barrier (BBB). Brain [5-HT] is generally higher in barramundi fed

a restricted ration than in those fed to satiation twice per day, suggesting a TRP or 5-HT negative feedback loop. Surprisingly no differences in the rate of cannibalistic mortality were observed between fish fed the 100 % ration and the 50 % ration contrary to the bulk of literature on intracohort fish cannibalism. High stress SWT of Atlantic salmon smolts led to an increase in brain [TRP] in the reference fed group, and to elevated serum cortisol and osmolality across all groups.

5.2 Aggressive behaviour

The current experiments show a lack of modulation in the display of aggressive behaviours by juvenile barramundi fed a TRP supplemented feed. Some behaviours exhibited by fish fed TRP supplemented feed were recorded to be different to those displayed by control groups at certain timepoints. It is suggested that these results are treated with caution however, as differences were at times conflicting, were few and far between, and often weakly significant at a 95% confidence interval . This result is contrary to responses to supplementary dietary TRP observed by most other researchers in other teleost fish. While behaviour was not a focus of the experiments in Chapters 3 and 4, observations were made whilst feeding and during routine husbandry tasks. It is accepted that these observations are qualitative and subject to error and bias, however no obvious signs of disparate behaviour, other than response to food, were noted between any of the feed treatments in either of the experiments.

No differences in initial lengths or weights of fish within each tank were present between treatments at the start of any of the experiments, however they were at the conclusion. Growth depensation within groups of fish is largely considered to reflect unequal access to the food resource, and the amount of food intake commonly reflects social rank. As subordinate behaviour often leads to a reduced share of the meal, the subordinate individual grows comparatively slower than the dominant, and thus the ability to maintain an elevated position within the hierarchy decreases. So, fiercer, more aggressive Atlantic salmon are more likely to dominate the food resource in an aquaculture environment (Adams *et al.* 1998).

The presence of similar growth depensation for all groups of fish, irrespective of feed type, across the 3 experiments in Chapters 3 and 4, strongly suggests that aggressive behaviour was not modulated by TRP inclusion, or if it was, no effect on dominance hierarchies or access to food were observed. This observation is particularly pertinent for the experiments of Chapter 3 where barramundi were fed TRP supplemented feeds for the full duration of the experiments, and fish were randomly allocated to tanks immediately prior to commencement. In experiment 3, Chapter 3, fish were hand fed to satiation twice daily, and thus there had been very limited time for social hierarchies to be established prior to the delivery of TRP supplemented food. Social dominance is of course strongly affected by fighting ability and positive outcomes from agonistic interactions are strongly affected by previous outcomes; winners keep winning. A meta-analysis across different taxa found an asymmetry in the magnitude of winner/loser effects, with winners having a twofold chance of subsequent victories, while losers are five times less likely to win (Rutte *et al.* 2006). Furthermore loser effects have a more sustained duration, and can even be present in the absence of winner effects (Hsu *et al.* 2006; Rutte *et al.* 2006). It has been suggested there may be different causal mechanisms between winner and loser effects, with the longer duration of loser effect possibly mediated by serotonin (Oliveira *et al.* 2009). So, if winners keep winning then size sorting populations of barramundi should have no long term benefits, only a brief reprieve from the threat of cannibalism, and the postulated weakening of an associated chronic stress response, which may allow for a short period of relatively greater food intake.

This may also partially explain personal observations that agonistic interactions appear most numerous amongst mid-sized individuals, rather than the larger or smaller individuals. The presence of small number of larger conspecifics within a group of Atlantic salmon has been found to reduce the rate of aggressive interactions between smaller fish (Adams *et al.* 2000). This effect was not observed in rainbow trout however (Flood *et al.* 2012). It is possible that variations in behavioural response to size differences across a cohort are species specific. Furthermore these responses may correlate with the strength of dominance hierarchies and the impact of size on position within the hierarchy for each species. Personal observations also suggest a possible reduction in rates of agonistic interactions at the upper and lower ends of

the cohort, maybe as a result of persistent winners and persistent losers avoiding conflict amongst each other; whereas in the middle of the cohort, size grading keeps placing winners with losers, thus creating opportunity for conflict. This of course would suggest that the observed social dominance hierarchies, measured by relative size, were at least partially predetermined, and if this argument is followed to its logical conclusion, the very first interactions between larval barramundi are key to their future survival. Irrespective of the pathway toward social dominance and consequent growth depensation, supplementary dietary TRP did not appear to deliver a different outcome when compared to groups fed non-supplemented feed, unless up regulated brain serotonergic activity acted differentially on dominant and subordinate individuals to deliver an opposite, but functionally equal outcome, i.e. previously winning dominant fish were subdued and previously losing subordinate fish were stimulated to become winners.

5.3 Serotonergic response

The optimal dietary TRP inclusion for growth, i.e. the point at which growth is not inhibited by a lack of TRP, for many teleost species is around 6 mg.g food^{-1}, a figure which unsurprisingly reflects the rate at which it is present in common protein sources, particularly fishmeal. Whilst the amount of TRP converted to 5-HT in humans is only 1-2 % of ingested TRP (Brown 1994), in fish the range could be wider. The relationship between dietary inclusion of TRP and plasma [TRP] in fish, as well as plasma [TRP] and brain [TRP] are linear (Johnston *et al.* 1990). The current studies suggest that, at high dietary TRP inclusion, uptake across the BBB may be optimised at a 50 % reduced ration compared to a satiation ration in juvenile barramundi, as discussed later in this section.

Differential serotonergic response has been reported relative to social status and behavioural type in a number of species (Winberg and Nilsson 1993a; Nelson and Chiavegatto 2001; Koolhaas *et al.* 2007). Brain [5-HT] from control animals, recorded as ng.g.brain^{-1}, are also variable with approximate concentrations for Arctic char of 4300 (Winberg and Nilsson 1993b), and for rainbow trout of 2700 (Lepage *et al.* 2002) and 45 (Johnston *et al.* 1990). The current

studies recorded brain [5-HT] of 500, and 350 for Atlantic salmon pre-SWT and at final sampling respectively. Barramundi brain [5-HT] were measured as 750 and 1000 for experiments 3 and 4 of Chapter 3 respectively. These differences in brain [5-HT] between species may reflect different coping styles, comparative behavioural types, level of food intake relative to satiation, ongoing exposure to environmental stressors, or could reflect limits of analytical detection.

The majority of research on dietary TRP induced fluctuations in serotonergic activity, describe very stable brain [5-HT] despite chronically high brain [TRP] suggesting that the turnover of 5-HT, rather than brain [5-HT] per se, is responsible for reported differences in HPI activation, food intake, and behaviour. Serotonergic activity, reported as the ratio of 5OH-IAA to 5-HT, is therefore strongly influenced by brain [5OH-IAA], which appears to have a stronger linear relationship with chronically elevated brain [TRP] than brain [5-HT]. A fluctuation in brain [5-HT] however, as observed in the brains of barramundi fed TRP supplemented feed at a restricted ration (Chapter 3, exp 4), can lead to potentially misleading conclusions by reporting the ratio of 5OH-IAA to 5-HT. Barramundi fed a 50 % restricted ration of feed supplemented with TRP to a total inclusion of between 14.8 and 19.0 $mg.g^{-1}$ showed a trend for increased brain [5-HT], and in the case of the feed with the highest amount (19.0$mg.g^{-1}$), brain [5-HT] was greater than for fish on the same feed but at a satiation ration. Relative to the satiation ration the TRP : LNAA in the feeds is the same; this might indicate preferential utilisation of TRP for growth when ration is limited, or it may reflect the longer duration of circulating LNAA's following a satiation ration. The proportion of protein in the feed has a strong effect on both brain [TRP] and [5-HT]; the higher the inclusion of protein, the greater the competition. As all dietary proteins are richer in other LNAAs than in TRP, and therefore the plasma : TRP ratio is reduced, decreasing transport across the BBB and conversion to 5-HT (Wurtman 1994). The feeds delivered to salmon and barramundi in the experiments of Chapters 3 and 4 had very similar protein levels, and thus differences in brain [5-HT] between species in the current study can't be attributed to protein inclusion, but suggest species specific differences possibly linked to relative daily food intake.

Brain [5OH-IAA] in control animals were broadly consistent but ranged from approximately 175 ng.g brain^{-1} in barramundi fed either a satiation ration or a 50 % ration (Exp 4, Chapter 3), to 110 ng.g brain^{-1} in barramundi fed a satiation ration (Exp 3, Chapter 3), and the lowest recorded was for Atlantic salmon, 60 ng.g brain^{-1}, also fed to satiation. The differences present between species are probably less important than the similarities in the relationship between [5OH-IAA] and [5-HT]. Brain [5OH-IAA] were very similar between rations (Exp 4, Chapter 3) and, as in all experiments, exhibited a linear relationship with TRP inclusion. This similarity in response between rations combined with the different brain [5-HT] response to TRP dose gives rise to a situation where 5OH-IAA : 5-HT is no different between feeds at the 50 % ration.

So, there is more TRP and 5-HT in the brain at the lower ration than at the satiation ration however the same amount of 5OH-IAA. This produces a generally lower, and equal between treatments, 5OH-IAA : 5-HT. One possible explanation for this is that increasing brain [TRP] within their physiologic range delivers a proportionate increase in brain [5-HT] (Schaechter and Wurtman 1989), however at brain TRP levels well beyond the normal range, the firing frequencies of the 5-HT releasing neurons is substantially decreased, as is the case in rats (Wurtman 1988). This negative feedback loop prevents brain [5-HT] from acute variation, suggesting adverse consequences to acute serotonin spikes.

These data provide apparently contrary explanations. Brain 5HO-IAA : 5-HT are greater in barramundi fed TRP supplemented feeds than the reference feed at a satiation ration however not at a 50 % ration, suggesting a faster turnover 5-HT at satiation, and thus presumably more explicit behavioural and HPI responses. The comparatively reduced and equal brain [TRP] recorded at satiation, compared to a 50 % ration, may be the result of that faster serotonergic activity, thereby depleting the available brain TRP sooner, which itself may be transported across the BBB at no faster rate than in the 50 % ration, due to LNAA competition. Alternatively, at the 50 % ration, the lack of inhibition of the firing frequency of the 5-HT releasing neurons has resulted in elevated brain [TRP] and [5-HT], and thus presumably more explicit behavioural and HPI responses.

Behavioural responses per se were not examined in the experiments of either Chapters 3 or 4 however physiological stress responses were, as was survival, an indicator of aggressive behaviour, for the experiments involving barramundi. Dietary TRP supplementation did not affect the physiological stress response at any inclusion in either barramundi or Atlantic salmon. The association between the HPI axis and the serotonergic system is well documented across taxa, so this result might be considered unusual, however the neurotransmitter analyses were conducted after a sustained period (50 days for barramundi) of exposure to TRP supplemented feeds, and thus the stress response may have returned to basal. In the case of the Atlantic salmon there was no indication of an elevated stress response to TRP supplemented feeds after 7 days of ingestion. There was also no difference in survival of barramundi between ration treatments (Exp 4, Chapter 3), which is contrary to expectation. All of the literature on cannibalism among groups of same initial size fish shows that limiting food supply results in higher intracohort predation. This is via greater competition for food, the establishment of dominance hierarchies and the subsequent growth depensation allowing for cannibalism to occur. Without the presence of size variation allowing for type 2 cannibalism, rates of cannibalism would not differ between treatments. It is not possible to attribute this better-than-expected survival at restricted ration to the behaviourally modulatory effects of supplementary TRP however, as there was no difference in survival between supplemented feeds and the reference feed. Neither were biologically relevant differences in behaviour noted between barramundi pairs (Exp 1, Chapter 2) and these fish were fed a daily ration very close to the 50 % ration delivered in Exp 4, Chapter 3. Whilst not statistically comparable these results would suggest that though supplementary dietary TRP leads to elevated brain [5-HT] at a restricted ration, and elevated serotonergic turnover (5HO-IAA ; 5-HT) at satiation, neither of these factors contribute to behavioural modulation or reduction in cannibalism.

5.4 Stress physiology

Elevated cortisol is implicated in numerous conditions of concern to commercial aquaculture: feeding depression, reduced locomotor activity, modified shoaling / schooling, poorer FCR's,

greater size variance, reduced reproductive performance and increased incidence of disease and mortality (Ellis *et al.* 2012). The effects of agonistic behaviour, such as the formation of dominance hierarchies, involve aggression, include elevated stress responses, and are thought to be responsible for considerable production losses in many fish culturing systems (Winberg and Nilsson 1993a). Unsuccessful transfers of salmon to saltwater have resulted in the transfer-associated cortisol peak being maintained, probably as a result of failed homeostatic mechanisms (Franklin *et al.* 1992). However, hypothalamic CRH activation and subsequent cortisol production should not necessarily be viewed as a harmful process; during smoltification benefits are conferred for Atlantic salmon from naturally occurring elevated cortisol, and treatment with cortisol prior to SWT has been shown to enhances hypo-osmoregulatory capacity in salmonids (Madsen 1990; Fuentes *et al.* 1996).

HPI axis activation has been linked with both catecholaminergic and serotonergic activity in salmonids (Winberg and Nilsson 1993a). Administration of TRP supplemented feed delivered an increased cortisol response in unstressed fish and a reduced cortisol response in stressed fish (Lepage *et al.* 2002). Acute stressors administered to rainbow trout for as little as 15 seconds have been shown to immediately elevate brain 5HT:5HIAA and for the effect to remain for 4 hours (Gesto *et al.* 2013). During chronic stress, plasma cortisol levels have been observed to return to resting levels despite continued response to the stressor (Vijayan and Leatherland 1990). Smoltification in salmonids, a process associated with increased plasma cortisol levels (Specker and Schreck 1982; Mommsen *et al.* 1999), and known in Chapter 3 to have occurred from osmolality data, is a plausible causal factor in elevated serotonergic activity observed at pre-SWT sampling (as smoltification is instigated prior to SWT). It is also possible that the cortisol spike associated with SWT, subdued by the time of post-SWT sampling, may have also triggered an up-regulation of serotonergic activity. Circulating corticosteroid response across all experiments of the current thesis in both Atlantic salmon and barramundi fed reference feeds were consistent. They reflected expected basal concentrations in unstressed fish, and elevations within an expected range for salmon post-SWT. There was no effect of dietary TRP supplementation on physiological stress response suggesting that the effect of stress on serotonergic activity as observed by Gesto (2013) is not reciprocal. This appears contrary to the

findings of Lepage (2002), as no differences in serum cortisol concentrations were observed between high stress SWT involving the draining of the tank and subsequent re-filling with seawater, or low stress SWT involving the flooding of the freshwater system with seawater. The lack of an administered stressor in Experiments 3 and 4 of Chapter 3, precludes comparative analysis of TRP mediated stress response relative to stressor. However the lack of difference in response between all treatments suggests it is unlikely. Neither of the experiments of Chapter 2 examined this possibility. There was however a sustained increase in brain [TRP] as a result of high stress SWT for Atlantic salmon fed the reference feed which is in accordance with other findings showing that stress increases brain [TRP] (Winberg and Nilsson 1993a). Overall the current experiments show no evidence of an elevation in circulating corticosteroids as a result of TRP supplementation of feed. Sampling occurred at 50 days post-commencement of TRP supplemented feeding for barramundi and it is therefore possible that any physiological stress response had moderated by this time. The shorter timeframe between commencement of feeding and blood sampling of 7 days for Atlantic salmon in Chapter 4 also showed no physiological stress response that could be attributed to supplementary dietary TRP. Stress responses in Atlantic salmon were evident after 9 days of TRP supplemented feed however were attributed to SWT rather than to dietary TRP. To appropriately test for a cortisol peak associated with supplementary dietary TRP it is suggested that sampling is conducted in the hours following an initial ration of supplemented feed.

5.5 Hypophagia

The regulation of food intake is complex and multidimensional. In mammals brain monoaminergic systems perform a role in modulation of food intake (Valassi *et al.* 2008; Feijo F *et al.* 2011). Serotonin is a monamine neurotransmitter and the activation of serotonergic neurons generally correlates with reduced food intake. The regulation of food intake in fish is via a similar process with similar effects (De Pedro *et al.* 1998a; Soengas 2014).

Hypophagia was only evident in both barramundi and Atlantic salmon when offered food to satiation twice daily. The mechanism involved in reduced food intake was considered to be

endocrine rather than an issue of palatability. Palatability concerns taste and texture of feed, and a response would be expected sooner than was evident, especially for the barramundi in 30°C water and consuming 14% of body weight per day. Fish would have substantially cleared the intestinal tract of the a.m. meal by the time of the p.m. meal and thus, if there was any aspect of the food that was unpalatable due to oesophageal taste buds, or a negative texture response, it is expected that refusal of food would be sooner.

There was no hypophagic response in barramundi fed feed containing 19.4 mg.g.TRP^{-1} at a ration of 8 % (a recommended ration for fish of this size), of body weight per day, which is approximately 1.5 mg TRP per g body weight. This suggests that either the dose was insufficient to suppress hunger, or that if hunger was suppressed, the effect was overcome within a 24 hour period, as fish were fed once daily each morning. Only the highest TRP inclusion (46.3 mg.g^{-1}) triggered a reduction in food intake in the salmon, however no inclusions between 21.8 mg.g^{-1} and 46.3 mg.g^{-1} were tested so a more precise point at which hypophagia becomes evident isn't known.

The average weight of the salmon fed a TRP supplemented feed at 21.8 mg.g^{-1} and 46.3 mg.g^{-1} after 7 days (the closest available time) of presentation with this feed was 119.45 g and 111.35 g respectively. The average respective food intake over this 7 day period was 1.07 g. fish^{-1}.day^{-1} and 0.68 g. fish^{-1}.day^{-1}. Using these data to calculate an approximation of the amount of ingested TRP required to deliver a hypophagic reaction in salmon is between 0.20 and 0.28 mg.g body weight^{-1}. Barramundi also exhibited hypophagia at 24h post first TRP supplemented feed at a similar rate of inclusion; between 23.8 mg.g^{-1} and 28.0 mg.g^{-1}. A similar calculation for barramundi showed that hypophagia on day 2 occurred at between 2.3 and 2.5 mg TRP.g body weight^{-1}. The tenfold difference between species is attributed predominately to the difference in food intake as a percentage of body weight: while the salmon consumed approximately 1% of body weight per day in food, the barramundi consumed approximately 12 %. This extremely wide variation in TRP consumption relative to body weight between barramundi and salmon, suggests that the similar hypophagic response results from 5-HT, not TRP. Brain [5-HT] was

elevated in both barramundi and salmon fed TRP supplemented feeds, however concentrations were more uniform than brain TRP and were consistent between species.

The differences in food intake as a percentage of body weight, both between species and more importantly at different life stages (size) of individuals, strongly supports that the passage of TRP across the BBB is restricted by other LNAA's. Consequently, when considering the effects of increased brain [TRP], [5-HT] or its metabolites, the total amount of TRP ingested is less significant than the ratio of TRP to other LNAA's. Whilst the mechanism of supplementary TRP mediated hypophagia is most likely consistent across species and taxa, the required dose to produce an effect appears highly variable, as does the duration and consistency of the effect.

The salmon in Chapter 4 exhibited a reduction in food intake within 24 hours of being presented with a TRP supplemented feed, and food intake was reduced by approximately 80 % after 3 days, however regained to approximately 65% of previous after 6 days of delivery possibly the result of re-conditioning to modified feed following food neophobia (the 'fear' of new food). The response in barramundi was much more consistent and sustained at both the lowest level that observed a hypophagic response at 24 hours and also at the level of inclusion most similar to that of the salmon. In fact it was 30 days until barramundi fed food containing TRP at 40.9 $mg.g^{-1}$ resumed food intake at a level consistently above 65 % of food intake on day 1, and at no point over the 50 day study was food intake greater than on day 1. As the hypophagic response to supplementary dietary TRP is different between species, it is suggested that a measure of mg ingested per g of body weight may be the most appropriate metric for describing the point of hypophagia and one that is more easily comparable.

The mode of appetite suppression was not examined in the current experiments, however recent work in this area on rainbow trout using 5-HT agonists, has shown that the activation of the 5-HT2C and 5-HT1A receptors trigger a hypophagic response (Pérez Maceira *et al.* 2014; Pérez-Maceira *et al.* 2016). Perez-Maceira *et al.* (2016) suggest that 5-HT acting through 5-HT2C receptors activates POMC, CART and CRF neurons in the hypothalamus resulting in increased abundance of these anorectic peptides. This explanation provides some clarification to the differential hypophagic response to brain [5-HT] between fish species as a result of

ingesting supplementary TRP. It is also in agreement with other studies that have observed feeding depression and concurrent increases in brain [CRF] after exposure to environmental, physical and social stressors (Bernier and Craig 2005; Craig *et al.* 2005; Doyon *et al.* 2005; Ortega *et al.* 2005; Ortega *et al.* 2013).

5.6 Conclusion

Supplementation of fish food with TRP causes an increase in brain [TRP] and serotonergic activity. There is strong evidence in the literature for the effect of 5-HT on the modulation of aggressive behaviours and the moderation of the physiological stress response, across taxa, including in fish. The mode of action of suppressed food intake in salmon post-SWT has not been well researched however is commonly attributed to a physiological stress response. The doses and rations delivered in the current studies neither reduced the rate of agonistic interactions (barramundi), nor alleviated post-SWT feeding depression (Atlantic salmon).

Serotonin mediated behavioural modulation has been shown in the literature to reflect behavioural type. Attempts to identify behavioural type in barramundi in this study (Chapter 1, Exp 2) were unsuccessful and thus it is possible, as suggested, that previously aggressive fish became less so and previously subordinate fish became more so after consuming TRP supplemented food. The exact, yet opposite outcome from this result appears unlikely however. Supplementary dietary TRP did negatively impact food intake, FCR and growth rate in barramundi over a 50 day period, however the rate of growth depensation and cannibalism were not affected. Over the same 50 day period ration size understandably affected growth rate, but interestingly not FCR or cannibalism.

There was no effect of TRP supplemented feed on the physiological stress response measured by blood cortisol, glucose or lactate concentrations. This suggests that the treatment itself was not a stressor for the fish, but also that there was no TRP mediated alleviation of stress response to stressors such as SWT. Neither physiological stress response nor food intake was different between fish subjected to high or low stress SWT.

While aquafeeds provide a suitable medium for the delivery of specific ingredients to cultured fish species, and the effects of dietary TRP on the serotonergic system are well established, the pathways that deliver observed effects remain incompletely understood. The development of functional feeds aimed at suppressing aggressive interactions between juvenile barramundi, or at limiting the stress response and feeding depression associated with SWT for Atlantic salmon smolts, via supplementary dietary TRP at the doses delivered in the current study, is not recommended.

Since 2000 much work has been conducted on fish using serotonin agonists and antagonists, and these studies have demonstrated the link between the up- or down-regulation of the serotonergic system, and further effects such as behavioural modulation, HPI axis response, or hypophagia. Some of the more recent research on the effect of the serotonergic system on hypophagia in fish, has highlighted some differences in action between fish (rainbow trout) and other vertebrates, while identifying specific receptors involved. Furthermore, it appears possible, certainly in the case of hypophagia, and possibly in the cases of behavioural modulation and HPI axis activation, that 5-HT may be less causal than previously thought, and that the serotonin mediated activation of other pathways, and consequent synthesis of other peptides, may have a more direct control.

The negative effects of behavioural interactions, such as injury, mortality, development of hierarchies, and subordination persist within the aquaculture environment and lead directly to poor utilisation of the feed resource, under performance, increased stress, and suppressed immunoreactivity. The reduction in return on investment on feed, loss of fish and growth during production, and the impact of growth depensation on harvest value, is a significant loss to business. Whilst behavioural observations provide something of a window into the overall health and welfare of the population, significant work needs to be conducted in the future on pathways toward reducing the rate and severity of the outcome of aggressive interactions. Behavioural studies remain somewhat hamstrung by the requirement of significant hours devoted to video analysis. When effective tracking and action recognition software become available, significant inroads into hierarchy dynamics will undoubtedly deliver welfare benefits

for farmed fish. Extensive work on the understanding of the neural pathways that govern food intake and behavioural response are needed to better understand why fish respond to situations in the way they do, and to be able to mitigate that response.

The impacts on welfare and production from cannibalism in barramundi and a reduction of food intake in post-SWT Atlantic salmon remain topics of interest. Future lines of enquiry:

- Genetic identification of fast growing, non-aggressive (non-cannibalizing) barramundi
- Serotonergic response between captive and wild fish as welfare indicator
- Evaluation of possible measures to deliver non-fatal brain serotonergic activity as welfare indicator
- Time course of serotonergic response and cortisol response over duration of smoltification using serotonin re-uptake inhibitors
- Assessment of bloating / stomach distension as factor in feeding depression at SWT

6 References

Abbott, J. C. and Dill, L., M. (1989). The relative growth of dominant and subordinate juvenile steelhead trout (*Oncorhynchus mykiss*) fed equal rations. Behaviour **108**(1/2): 104-113.

Adams, C., Huntingford, F., Turnbull, J., Arnott, S. and Bell, A. (2000). Size heterogeneity can reduce aggression and promote growth in Atlantic salmon parr. Aquaculture International **8**(6): 543-549.

Adams, C. E. and Huntingford, F. A. (1996). What is a successful fish? Determinants of competitive success in Arctic char (*Salvelinus alpinus*) in different social contexts. Canadian Journal of Fisheries and Aquatic Sciences **53**(11): 2446-2450.

Adams, C. E., Huntingford, F. A., Turnbull, J. F. and Beattie, C. (1998). Alternative competitive strategies and the cost of food acquisition in juvenile Atlantic salmon (Salmo salar). Aquaculture **167**(1): 17-26.

Alanärä, A., Winberg, S., Brännäs, E., Kiessling, A., Höglund, E. and Elofsson, U. (1998). Feeding behaviour, brain serotonergic activity levels, and energy reserves of Arctic char (*Salvelinus alpinus*) within a dominance hierarchy. Canadian Journal of Zoology **76**(2): 212-220.

Aldegunde, M., Garcia, J., Soengas, J. L. and Rozas, G. (1998). Uptake of tryptophan into brain of rainbow trout (*Oncorhynchus mykiss*). Journal of Experimental Zoology **282**(3): 285-289.

Aldegunde, M., Soengas, J. L. and Rozas, G. (2000). Acute effects of L-tryptophan on tryptophan hydroxylation rate in brain regions (hypothalamus and medulla) of rainbow trout (*Oncorhynchus mykiss*). J Exp Zool **286**(2): 131-135.

Alexander, G., Sweeting, R. and Mckeown, B. (1994). The shift in visual pigment dominance in the retinae of juvenile coho salmon (*Oncorhynchus kisutch*): an indicator of smolt status. Journal of Experimental Biology **195**(1): 185-197.

Alvarenga, C. and Volpato, G. (1995). Agonistic profile and metabolism in alevins of the Nile Tilapia. Physiology and Behaviour **57**(1): 75-80.

Appelbaum, S. and Arockiaraj, A. J. (2010). Sibling cannibalism in juvenile Asian sea bass (*Lates calcarifer*) reared under different photoperiods. AACL Bioflux **3**(5): 384-392.

Applebaum, S. and Arockiaraj, A., J. (2010). Sibling cannibalism in juvenile Asian sea bass (*Lates calcarifer*) reared uner different photoperiods. AACL Bioflux **3**(5).

Aragao, C., Corte-Real, J., Costas, B., Dinis, M. T. and Conceicao, L. E. C. (2008). Stress response and changes in amino acid requirements in Senegalese sole (*Solea senegalensis*). Amino Acids **34**(1): 143-148.

Aragao, C., Costas, B., Vargas-Chacoff, L., Ruiz-Jarabo, I., Dinis, M. T., Miguel Mancera, J. and Conceicao, L. E. C. (2010). Changes in plasma amino acid levels in a euryhaline fish exposed to different environmental salinities. Amino Acids **38**(1): 311-317.

Arockiaraj, A. J. and Appelbaum, S. (2011). Sibling cannibalism in juvenile barramundi, *Lates calcarifer* (actinopterygii: perciformes: centropomidae), reared under different light conditions. Acta Ichthyologica Et Piscatoria **41**(1): 7-11.

Ashley, P. J. (2007). Fish welfare: Current issues in aquaculture. Applied Animal Behaviour Science **104**(3 - 4): 199 - 235.

Bakker, T. C. M. (1986). Aggressiveness in Sticklebacks (*Gasterosteus aculeatus* L.): A Behaviour-Genetic Study. Behaviour **98**(1/4): 1-144.

Bale, T. L., Contarino, A., Smith, G. W., Chan, R., Gold, L. H., Sawchenko, P. E., Koob, G. F., Vale, W. W. and Lee, K. F. (2000). Mice deficient for corticotropin-releasing hormone receptor-2 display anxiety-like behaviour and are hypersensitive to stress. Nature Genetics **24**(4): 410-414.

Barlow, C. G., Pearce, M. G., Rodgers, L. J. and Clayton, P. (1995). Effects of photoperiod on growth, survival and feeding periodicity of larval and juvenile barramundi, *Lates calcarifer* (Bloch). Aquaculture **138**(1-4): 159-168.

Barrows, F. T. and Frost, J. B. (2014). Evaluation of the nutritional quality of co-products from the nut industry, algae and an invertebrate meal for rainbow trout, *Oncorhynchus mykiss*. Aquaculture **434**: 315-324.

Barton, B. A., Schreck C. B and Barton, L. D. (1987a). Effects of chronic cortisol administration and daily acute stress on growth, physiological conditions, and stress responses in juvenile rainbow trout. Dis. aquat. Org **2**: 173-185.

Barton, B. A., Schreck C. B. and Barton L, D. (1987b). Effects of chronic cortisol administration and daily acute stress on growth, physiological conditions, and stress responses in juvenile rainbow-trout. Dis. aquat. Org **2**: 173 - 185.

Barton, B. A., Schreck, C. B., Ewing, R. D., Hemmingsen, A. R. and Patiño, R. (1985). Changes in plasma cortisol during stress and smoltification in Coho Salmon, *Oncorhynchus kisutch*. General and Comparative Endocrinology **59**(3): 468-471.

Beacher, F. D. C. C., Gray, M. A., Minati, L., Whale, R., Harrison, N. A. and Critchley, H. D. (2011). Acute tryptophan depletion attenuates conscious appraisal of social emotional signals in healthy female volunteers. Psychopharmacology **213**(2): 603-613.

Bejo Wolkers, C. P., Serra, M., Hoshiba, M. A. and Urbinati, E. C. (2012). Dietary L-tryptophan alters aggression in juvenile matrinxa, *Brycon amazonicus*. Fish Physiology and Biochemistry **38**(3): 819-827.

Bernier, N. J. (2006). The corticotropin-releasing factor system as a mediator of the appetite-suppressing effects of stress in fish. General and Comparative Endocrinology **146**(1): 45-55.

Bernier, N. J., Bedard, N. and Peter, R. E. (2004). Effects of cortisol on food intake, growth, and forebrain neuropeptide Y and corticotropin-releasing factor gene expression in goldfish. General and Comparative Endocrinology **135**(2): 230-240.

Bernier, N. J. and Craig, P. M. (2005). CRF-related peptides contribute to stress response and regulation of appetite in hypoxic rainbow trout. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology **289**(4): R982-R990.

Bernier, N. J. and Peter, R. E. (2001a). Appetite-suppressing effects of urotensin I and corticotropin-releasing hormone in goldfish (*Carassius auratus*). Neuroendocrinology **73**(4): 248-260.

Bernier, N. J. and Peter, R. E. (2001b). The hypothalamic–pituitary–interrenal axis and the control of food intake in teleost fish. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology **129**(2–3): 639-644.

Best, J., Nijhout, H. F. and Reed, M. (2010). Serotonin synthesis, release and reuptake in terminals: a mathematical model. Theoretical Biology and Medical Modelling **7**(1): 1-26.

Blanchard, D. C. and Blanchard, R. J. (1988). Ethoexperimental approaches to the biology of emotion. Annual Review of Psychology **39**: 43-68.

Blazer, V. S. (1992). Nutrition and disease resistance in fish. Annual Review of Fish Diseases **2**(0): 309-323.

Blumstein, D. T., C., D. J. and Evans, C. S. (2006). *JWatcher 1.0.* Available from http;//www.jwatcher.ucla.edu.

Blundell, J. E. and Halford, J. C. G. (1998). Serotonin and appetite regulation: Implications for the pharmacological treatment of obesity. CNS Drugs **9**(6): 473-495.

Boeuf, G. (1993). Salmonid Smolting: a pre-adaption to the oceanic environment. Fish Ecophysiology. J. Rankin, C., and F. Jensen, B. London, Chapman and Hall**:** 105-135.

Bolhuis, J. E., Parmentier, H. K., Schouten, W. G. P., Schrama, J. W. and Wiegant, V. A. (2003). Effects of housing and individual coping characteristics on immune responses of pigs. Physiology & Behavior **79**(2): 289-296.

Bosmans, J. M. P., Schipp, G. R., Gore, D. J., Jones, B., Vauchez, F. E. and Newman, K. K. (2004). Early weaning of barramundi, *Lates calcarifer* (Bloch), in a commercial, intensive, semi-automated, recirculated larval rearing system. The second hatchery feeds and technology workshop, Novatel Century, Sydney, Australia.

Bridges, C. D. B. and Delisle, C. E. (1974). Brief observations concerning the visual pigments of some selected fishes from Lake Heney, Quebec, a relict of glacial Lake Gatineau. Vision Research **14**(2): 187-193.

Brown, G. L., Goodwin, F. K., Ballenger, J. C., Goyer, P. F. and Major, L. F. (1979). Aggression in humans correlates with cerebrospinal fluid amine metabolites. Psychiatry Research **1**(2): 131-139.

Brown, R., R. (1994). Tryptophan Metabolism: A Review. L-Tryptophan: Current Prospects in Medicine and Drug Safety. W. Kochen and H. Steinhart. Berlin, Walter de Gruyter**:** 17 - 30.

Budaev, S., V (1997). The statistical analysis of behavioural latency measures. ICSP Newsletter **14**: 1-4.

Carter, C. G., McCarthy, I. D., Houlihan, D. F., Johnstone, R., Walsingham, M. V. and Mitchell, A. I. (1994). Food consumption, feeding behaviour, and growth of triploid and diploid Atlantic salmon, Salmo salar L., parr. Canadian Journal of Zoology **72**(4): 609-617.

Caspi, A., Sugden, K., Moffitt, T. E., Taylor, A., Craig, I. W., Harrington, H., McClay, J., Mill, J., Martin, J., Braithwaite, A. and Poulton, R. (2003). Influence of life stress on depression: Moderation by a polymorphism in the 5-HTT gene. Science **301**(5631): 386.

Chiao, J. Y. (2010). Neural basis of social status hierarchy across species. Current Opinion in Neurobiology **20**(6): 803-809.

Clotfelter, E. D., O'Hare, E. P., McNitt, M. M., Carpenter, R. E. and Summers, C. H. (2007). Serotonin decreases aggression via 5-HT1A receptors in the fighting fish, *Betta splendens*. Pharmacology Biochemistry and Behavior **87**(2): 222-231.

Coleman, K. and Wilson, D. S. (1998). Shyness and boldness in pumpkinseed sunfish: individual differences are context-specific. Animal Behaviour **56**(4): 927-936.

Coloso, R. M., Murillo-Gurrea, D. P., Borlongan, I. G. and Catacutan, M. R. (2004a). Tryptophan requirement of juvenile Asian sea bass Lates calcarifer. Journal of Applied Ichthyology **20**(1): 43-47.

Coloso, R. M., Murillo-Gurrea, D. P., Borlongan, I. G. and Catacutan, M. R. (2004b). Tryptophan requirement of juvenile Asian sea bass, *Lates calcarifer*. Journal of Applied Ichthyology **20**(1): 43-47.

Conceicao, L. E. C., Aragao, C., Dias, J., Costas, B., Terova, G., Martins, C. and Tort, L. (2012). Dietary nitrogen and fish welfare. Fish Physiology and Biochemistry **38**(1): 119-141.

Costas, B., Aragao, C., Mancera, J. M., Dinis, M. T. and Conceicao, L. E. C. (2008). High stocking density induces crowding stress and affects amino acid metabolism in Senegalese sole, *Solea senegalensis* (Kaup 1858) juveniles. Aquaculture Research **39**(1): 1-9.

Costas, B., Aragão, C., Ruiz-Jarabo, I., Vargas-Chacoff, L., Arjona, F., Mancera, J., Dinis, M. and Conceição, L. C. (2012a). Different environmental temperatures affect amino acid metabolism in the eurytherm teleost Senegalese sole (*Solea senegalensis*) Kaup, 1858 as indicated by changes in plasma metabolites. Amino Acids **43**(1): 327-335.

Costas, B., Aragao, C., Soengas, J. L., Miguez, J. M., Rema, P., Dias, J., Afonso, A. and Conceicao, L. E. C. (2012b). Effects of dietary amino acids and repeated handling on stress response and brain monoaminergic neurotransmitters in Senegalese sole (*Solea senegalensis*) juveniles. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology **161**(1): 18-26.

Costas, B., Conceicao, L. E. C., Aragao, C., Martos, J. A., Ruiz-Jarabo, I., Mancera, J. M. and Afonso, A. (2011). Physiological responses of Senegalese sole (*Solea senegalensis* Kaup, 1858) after stress challenge: Effects on non-specific immune parameters, plasma free amino acids and energy metabolism. Aquaculture **316**(1-4): 68-76.

Coste, S. C., Heard, A. D., Phillips, T. J. and Stenzel-Poore, M. P. (2006). Corticotropin-releasing factor receptor type 2-deficient mice display impaired coping behaviors during stress. Genes, Brain and Behavior **5**(2): 131-138.

Craig, P. M., Al-Timimi, H. and Bernier, N. J. (2005). Differential increase in forebrain and caudal neurosecretory system corticotropin-releasing factor and urotensin I gene expression associated with seawater transfer in rainbow trout. Endocrinology **146**(9): 3851-3860.

Cubitt, K. F., Winberg, S., Huntingford, F. A., Kadri, S., Crampton, V. O. and Overli, O. (2008). Social hierarchies, growth and brain serotonin metabolism in Atlantic salmon (*Salmo salar*) kept under commercial rearing conditions. Physiology and Behavior **94**(4): 529-535.

Culley, W. J., Jolly, D. H., Mertz, E. T. and Saunders, R. N. (1963). Effect of a tryptophan deficiant diet on brain serotonin and plasma tryptophan level. Proceedings of the Society for Experimental Biology and Medicine **113**(3): 645-&.

Curnow, J., King, J., Partridge, G. and Kolkovski, S. (2006). Effects of two commercial microdiets on growth and survival of barramundi (*Lates calcarifer* Bloch) larvae within various early weaning protocols. Aquaculture Nutrition **12**(4): 247-255.

Damsgard, B. and Arnesen, A. M. (1998). Feeding, growth and social interactions during smolting and seawater acclimation in Atlantic salmon, *Salmo salar* L. Aquaculture **168**(1-4): 7-16.

Damsgård, B. and Arnesen, A. M. (1998). Feeding, growth and social interactions during smolting and seawater acclimation in Atlantic salmon, Salmo salar L. Aquaculture **168**(1–4): 7-16.

Davidson, J., Barrows, F. T., Kenney, P. B., Good, C., Schroyer, K. and Summerfelt, S. T. (2016). Effects of feeding a fishmeal-free versus a fishmeal-based diet on post-smolt Atlantic salmon *Salmo salar* performance, water quality, and waste production in recirculation aquaculture systems. Aquacultural Engineering **74**: 38-51.

Davis, M. W. and Olla, B. L. (1987). Aggression and variation in growth of Chum salmon (*Oncorhynchus keta*) juveniles in seawater: Effects of limited ration. Canadian Journal of Fisheries and Aquatic Sciences **44**(1): 192-197.

Dawkins, M. S. (2004). Using behaviour to assess animal welfare. Animal Welfare **13**: S3-S7.

De Pedro, N., Alonso-Gómez, A. L., Gancedo, B., Delgado, M. J. and Alonso-Bedate, M. (1993). Role of corticotropin-releasing factor (CRF) as a food intake regulator in goldfish. Physiology & Behavior **53**(3): 517-520.

De Pedro, N., Alonso-Gomez, A. L., Gancedo, B., Valenciano, A. I., Delgado, M. J. and Alonso-Bedate, M. (1997). Effect of alpha-helical-CRF[9-41] on feeding in goldfish: involvement of cortisol and catecholamines. Behav Neurosci **111**(2): 398-403.

De Pedro, N., Pinillos, M. L., Valenciano, A. I., Alonso-Bedate, M. and Delgado, M. J. (1998a). Inhibitory effect of serotonin on feeding behavior in goldfish. Peptides **19**(3): 505-511.

de Pedro, N., Pinillos, M. L., Valenciano, A. I., Alonso-Bedate, M. and Delgado, M. J. (1998b). Inhibitory Effect of Serotonin on Feeding Behavior in Goldfish: Involvement of CRF. Peptides **19**(3): 505-511.

De Silva, S. S. (1995). Fish Nutrition in Aquaculture, London : Chapman and Hall, 1995.

De Silva, S. S. and Anderson, T. A. (1995). Nutrition in Aquaculture. London, Chapman & Hall.

De Simoni, M. G., Sokola, A., Fodritto, F., Toso, G. D. and Algeri, S. (1987). Functional meaning of tryptophan-induced increase of 5-HT metabolism as clarified by in vivo voltammetry. Brain Research **411**(1): 89-94.

Deckel, A. W. (1996). Behavioral changes in *Anolis carolinensis* following injection with fluoxetine. Behavioural Brain Research **78**(2): 175-182.

Deckel, A. W. and Fuqua, L. (1998). Effects of serotonergic drugs on lateralized aggression and aggressive displays in *Anolis carolinensis*. Behavioural Brain Research **95**(2): 227-232.

Desjardins, J. K. and Fernald, R. D. (2010). What do fish make of mirror images? Biology Letters **6**(6): 744-747.

Di-Poï, C., Attia, J., Bouchut, C., Dutto, G., Covès, D. and Beauchaud, M. (2007). Behavioral and neurophysiological responses of European sea bass groups reared under food constraint. Physiology & Behavior **90**(4): 559-566.

Dou, S.-Z., Masuda, R., Tanaka, M. and Tsukamoto, K. (2004). Size hierarchies affecting the social interactions and growth of juvenile Japanese flounder, *Paralichthys olivaceus*. Aquaculture **233**(1-4): 237-249.

Doyon, C., Trudeau V, L. and Moon T, W. (2005). Stress elevates corticotropin-releasing factor (CRF) and CRF binding protein mRNA levels in rainbow trout (*Oncorhynchus mykiss*). Journal of Endocrinology **186**: 123-130.

Drent, P. J., Oers, K. v. and Noordwijk, A. J. v. (2003). Realized heritability of personalities in the great tit (*Parus major*). Proceedings: Biological Sciences **270**(1510): 45-51.

Dugatkin, L. A. (1997). Winner and loser effects and the structure of dominance hierarchies. Behavioral Ecology **8**(6): 583-587.

Dunn, A., J., and Berridge, C., W. (1990). Physiological and behavioural responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety or stress responses? Brain Reseach Reviews **15**: 71-100.

Duray, M., N and Juario, J., V (1988). Broodstock management and seed production of the rabbitfish, *Siganus guttatus* (Bloch) and the sea bass, *Lates calcarifer*. Perspectives in Aquaculture Development in Southeast Asia and Japan. V. Juario J and L. Benitez, V. Aquaculture Department of the Southeast Asian Fisheries Development Center, Iloilo, Phillipines**:** 195 - 210.

Ebbesson, L., O, E.,, Holmqvist, B., Ostholm, T. and Ekström, P. (1992). Transient serotonin-immunoreactive neurons coincide with a critical period of neural development in coho salmon (*Oncorhynchus kisutch)*. Cell Tissue Research **268**: 389-392.

Ebbesson, L. O. E., Björnsson, B. T., Ekström, P. and Stefansson, S. O. (2008). Daily endocrine profiles in parr and smolt Atlantic salmon. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology **151**(4): 698-704.

Ebbesson, L. O. E., Ekström, P., Ebbesson, S. O. E., Stefansson, S. O. and Holmqvist, B. (2003). Neural circuits and their structural and chemical reorganization in the light–brain–pituitary axis during parr–smolt transformation in salmon. Aquaculture **222**(1–4): 59-70.

Ebbesson, S. O. E., Smith, J., Co, C. and Ebbesson, L. O. E. (1996). Transient alterations in neurotransmitter levels during a critical period of neural development in coho salmon (*Oncorhyncus kisutch*). Brain Research **742**(1–2): 339-342.

Einum, S. and Fleming, I. A. (1997). Genetic divergence and interactions in the wild among native, farmed and hybrid Atlantic salmon. Journal of Fish Biology **50**(3): 634-651.

Elgar, M., A and Crespi, B., J (1992). Cannibalism: Ecology and Evolution Among Diverse Taxa. Oxford, Oxford University Press.

Ellis, T., North, B., Scott, A. P., Bromage, N. R., Porter, M. and Gadd, D. (2002). The relationships between stocking density and welfare in farmed rainbow trout. Journal of Fish Biology **61**(3): 493-531.

Ellis, T., Yildiz, H. Y., López-Olmeda, J., Spedicato, M. T., Tort, L., Øverli, O. and Martins, C. I. M. (2012). Cortisol and finfish welfare. Fish Physiology and Biochemistry **38**(1): 163-188.

FAO (2012). The State of World Fisheries and Aquaculture, 2012.

FAO (2014). The state of World Fisheries and Aquaculture 2014.

Farhangi, M. and Carter, C. G. (2001). Growth, physiological and immunological responses of rainbow trout (Oncorhynchus mykiss) to different dietary inclusion levels of dehulled lupin (*Lupinus angustifolius*). Aquaculture Research **32**: 329-340.

Feijo F, M., Bertoluci M, C. and C., R. (2011). Serotonin and hypopthalamic control of hunger: a review. Rev Assoc Med Bras **57**: 74-77.

Fernandes, W. P. A., Ibbotson, A. T., Griffiths, S. W., Maxwell, D. L., Davison, P. I. and Riley, W. D. (2015). Does relatedness influence migratory timing and behaviour in Atlantic salmon smolts? Animal Behaviour **106**: 191-199.

Fernstrom, J. D. and Fernstrom, M. H. (2007). Tyrosine, Phenylalanine, and Catecholamine Synthesis and Function in the Brain. The journal of Nutrition **137**(6).

Filby, A. L., Paull, G. C., Hickmore, T. F. A. and Tyler, C. R. (2010). Unravelling the neurophysiological basis of aggression in a fish model. Bmc Genomics **11**.

Flik G, Klaren P. H. M, Van den Burg E. H, Metz J. R and O., H. M. (2006). CRF and stress in fish. General and Comparative Endocrinology(146): 36-44.

Flood, M. J., John Purser, G. and Carter, C. G. (2011). The effects of changing feeding frequency simultaneously with seawater transfer in Atlantic salmon *Salmo salar* L. smolt. Aquaculture International **20**(1): 29-40.

Flood, M. J., Noble, C., Kagaya, R. and Tabata, M. (2012). Can the presence of a large conspecific improve the production and welfare of groups of smaller self-feeder competent rainbow trout? Aquaculture Research **43**(11): 1620-1627.

Folmar, L. C. and Dickhoff, W. W. (1980). The parr—Smolt transformation (smoltification) and seawater adaptation in salmonids: A review of selected literature. Aquaculture **21**(1): 1-37.

Forster, I., Higgs, D. A., Dosanjh, B. S., Rowshandeli, M. and Parr, J. (1999). Potential for dietary phytase to improve the nutritive value of canola protein concentrate and decrease phosphorus output in rainbow trout (*Oncorhynchus mykiss*) held in 11 degrees C fresh water. Aquaculture **179**(1-4): 109-125.

Franklin, C. E., Forster, M. E. and Davison, W. (1992). Plasma cortisol and osmoregulatory changes in sockeye salmon transferred to sea water: Comparison between successful and unsuccessful adaptation. Journal of Fish Biology **41**(1): 113-122.

Fraser, T. W. K., Vindas, M. A., Fjelldal, P. G., Winberg, S., Thörnqvist, P.-O., Øverli, Ø., Skjæraasen, J.-E., Hansen, T. J. and Mayer, I. (2015). Increased reactivity and monoamine dysregulation following stress in triploid Atlantic salmon (*Salmo salar*). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology **185**: 125-131.

Fuentes, J., Bury, N. R., Carroll, S. and Eddy, F. B. (1996). Drinking in Atlantic salmon presmolts (*Salmo salar* L.) and juvenile rainbow trout (*Oncorhynchus mykiss* Walbaum) in response to cortisol and sea water challenge. Aquaculture **141**(1–2): 129-137.

Fuentes, J. and Eddy, F. B. (1997). Drinking in Atlantic salmon presmolts and smolts in response to growth hormone and salinity. Comparative Biochemistry and Physiology Part A: Physiology **117**(4): 487-491.

Gesto, M., López-Patiño, M. A., Hernández, J., Soengas, J. L. and Míguez, J. M. (2013). The response of brain serotonergic and dopaminergic systems to an acute stressor in rainbow trout: a time course study. Journal of Experimental Biology **216**(23): 4435-4442.

Giacalone, E., Tansella, M., Valzelli, L. and Garattini, S. (1968). Brain serotonin metabolism in isolated aggressive mice. Biochemical Pharmacology **17**(7): 1315-1327.

Gibson, R. J. (1983). Water velocity as a factor in the change from aggressive to schooling behaviour and subsequent migration of Atlantic salmon smolt (*Salmo salar*). Le Naturaliste Canadien **111**: 143-148.

Gjedrem, T., Robinson, N. and Rye, M. (2012). The importance of selective breeding in aquaculture to meet future demands for animal protein: A review. Aquaculture **350**: 117-129.

Glencross, B., Evans, D., Hawkins, W. and Jones, B. (2004). Evaluation of dietary inclusion of yellow lupin (*Lupinus luteus*) kernel meal on the growth, feed utilisation and tissue histology of rainbow trout (*Oncorhynchus mykiss*). Aquaculture **235**(1-4): 411-422.

Glencross, B., Hawkins, W. and Curnow, J. (2003). Evaluation of canola oils as alternative lipid resources in diets for juvenile red seabream, *Pagrus auratus*. Aquaculture Nutrition **9**(5): 305-315.

Glencross, B., Sweetingham, M. and Hawkins, W. (2010). A digestibility assessment of pearl lupin (*Lupinus mutabilis*) meals and protein concentrates when fed to rainbow trout (*Oncorhynchus mykiss*). Aquaculture **303**(1–4): 59-64.

Glencross, B. D. (2008). A factorial growth and feed utilization model for barramundi, *Lates calcarifer* based on Australian production conditions. Aquaculture Nutrition **14**(4): 360-373.

Glencross, B. D., Booth, M. and Allan, G. L. (2007). A feed is only as good as its ingredients - a review of ingredient evaluation strategies for aquaculture feeds. Aquaculture Nutrition **13**(1): 17-34.

Goddard, S. (1996). Feed Management in Intensive Aquaculture. New York, Chapman & Hall.

Goodson, J. L. (2005). The vertebrate social behavior network: Evolutionary themes and variations. Hormones and Behavior **48**(1): 11-22.

Gregory, T. R. and Wood, C., M. (1998). The effects of chronic plasma cortisol elevation on the feeding behaviour, growth, competitive ability, and swimming performance of juvenile rainbow trout. Physiological and Biochemical Zoology: Ecological and Evolutionary Approaches **72**(3): 286-295.

Halford, J. C. G., Harrold, J. A., Boyland, E. J., Lawton, C. L. and Blundell, J. E. (2007). Serotonergic Drugs: Effects on appetite expression and use for the treatment of obesity. Drugs **67**(1): 27-55.

Hart, P. J. B. (1993). Teleost foraging:Facts and theories. Behaviour of Teleost Fishes

T. J. Pitcher. London, Chapman & Hall.

Hecht, T. and Pienaar, A. G. (1993). A review of cannibalism and its implications in fish larviculture. Journal of the World Aquaculture Society **24**(2): 246-261.

Heinrichs, S. C., Lapsansky, J., Lovenberg, T. W., De Souza, E. B. and Chalmers, D. T. (1997). Corticotropin-releasing factor CRF1, but not CRF2, receptors mediate anxiogenic-like behavior. Regulatory Peptides **71**(1): 15-21.

Hickman, C. P., Roberts, L. S. and Larson, A. (1998). Biology of Animals. USA, McGraw-Hill.

Higley, J. D. (2001). Individual differences in alcohol-induced aggression. A nonhuman-primate model. Alcohol Res Health **25**(1): 12-19.

Higley, J. D., Mehlman, P. T., Poland, R. E., Taub, D. M., Vickers, J., Suomi, S. J. and Linnoila, M. (1996). CSF testosterone and 5-HIAA correlate with different types of aggressive behaviors. Biological Psychiatry **40**(11): 1067-1082.

Hirano, T. (1974). Some Factors Regulating Water Intake by the Eel, *Anguilla Japonica*. Journal of Experimental Biology **61**(3): 737-747.

Hoar, W. S. (1976). Smolt Transformation: Evolution, Behavior, and Physiology. Journal of the Fisheries Research Board of Canada **33**(5): 1233-1252.

Hoglund, E., Bakke, M. J., Overli, O., Winberg, S. and Nilsson, G. E. (2005). Suppression of aggressive behaviour in juvenile Atlantic cod (*Gadus morhua*) by L-tryptophan supplementation. Aquaculture **249**(1-4): 525-531.

Hoglund, E., Sorensen, C., Bakke, M. J., Nilsson, G. E. and Overli, O. (2007). Attenuation of stress-induced anorexia in brown trout (*Salmo trutta*) by pre-treatment with dietary L-tryptophan. British Journal of Nutrition **97**(4): 786-789.

Hokanson, K. E. F. and Lien, G. J. (1986). Effects of diet on growth and survival of larval walleyes. The Progressive Fish-Culturist **48**(4): 250-258.

Holstein, B. (1979). Gastric acid secretion and water balance in the marine teleost *Gadus morhua*. Acta Physiologica Scandinavica **105**(1): 93-107.

Hosfeld, C. D., Hammer, J., Handeland, S. O., Fivelstad, S. and Stefansson, S. O. (2009). Effects of fish density on growth and smoltification in intensive production of Atlantic salmon (*Salmo salar* L.). Aquaculture **294**(3–4): 236-241.

Houde, A. L. S., Fraser, D. J. and Hutchings, J. A. (2010). Reduced anti-predator responses in multi-generational hybrids of farmed and wild Atlantic salmon (Salmo salar L.). Conservation Genetics **11**(3): 785-794.

Hseu, J. R., Lu, F. I., Su, H. M., Wang, L. S., Tsai, C. L. and Hwang, P. P. (2003). Effect of exogenous tryptophan on cannibalism, survival and growth in juvenile grouper, *Epinephelus coioides*. Aquaculture **218**(1-4): 251-263.

Hsu, Y., Earley, R. L. and Wolf, L. L. (2006). Modulation of aggressive behaviour by fighting experience: mechanisms and contest outcomes. Biol Rev Camb Philos Soc **81**(1): 33-74.

Hsu, Y. and Wolf, L. L. (1999). The winner and loser effect: integrating multiple experiences. Anim Behav **57**(4): 903-910.

Huising, M. O., Metz, J. R., van Schooten, C., Taverne-Thiele, A. J., Hermsen, T., Verburg-van Kemenade, B. M. and Flik, G. (2004). Structural characterisation of a cyprinid (*Cyprinus carpio* L.) CRH, CRH-BP and CRH-R1, and the role of these proteins in the acute stress response. J Mol Endocrinol **32**(3): 627-648.

Huntingford, F., A, Metcalfe, N., B, Thorpe, J., E. and Graham, W., D (1989). Social dominance and body size in Atlantic salmon parr, *Salmo salar*. Journal of Fish Biology **36**(6): 877-881.

Huntingford, F. and Adams, C. (2005a). Behavioural syndromes in farmed fish: implications for production and welfare. Behaviour **142**: 1207-1221.

Huntingford, F. A., Adams, C., Braithwaite, V. A., Kadri, S., Pottinger, T. G., Sandoe, P. and Turnbull, J. F. (2006). Current issues in fish welfare. Journal of Fish Biology **68**(2): 332-372.

Huntingford, F. A. and Adams, C. A. (2005b). Behavioural syndromes in farmed fish: implications for production and welfare. Behaviour **142**: 1207-1221.

Huntingford, F. A. and Kadri, S. (2009). Taking account of fish welfare: lessons from aquaculture. Journal of Fish Biology **75**(10): 2862-2867.

Itoi, K., Seasholtz, A. F. and Watson, S. J. (1998). Cellular and extracellular regulatory mechanisms of hypothalamic corticotropin-releasing hormone neurons. Endocr J **45**(1): 13-33.

Iversen, M., Eliassen, R. A. and Finstad, B. (2009). Potential benefit of clove oil sedation on animal welfare during salmon smolt, *Salmo salar* L. transport and transfer to sea. Aquaculture Research **40**(2): 233-241.

Jobling, M., Boujard, T. and Houlihan, D. F. (2001). Food intake in fish, Oxford : Blackwell Science, 2001.

Jobling, M. and Koskela, J. (1996). Interindividual variations in feeding and growth in rainbow trout during restricted feeding and in a subsequent period of compensatory growth. Journal of Fish Biology **49**(4): 658-667.

Jobling, M. and Wandsvik, A. (1982). Effect of social interactions on growth rates and conversion efficiency of Arctic charr, *Salvelinus alpinus* L Journal of Fish Biology **22**(5): 577-584.

Johnsson, J. I., Höjesjö, J. and Fleming, I. A. (2001). Behavioural and heart rate responses to predation risk in wild and domesticated Atlantic salmon. Canadian Journal of Fisheries and Aquatic Sciences **58**(4): 788-794.

Johnsson, J. I., Jönsson, E. and Björnsson, B. T. (1996). Dominance, Nutritional State, and Growth Hormone Levels in Rainbow Trout (*Oncorhynchus mykiss*). Hormones and Behavior **30**(1): 13-21.

Johnston, W. L., Atkinson, J. L., Hilton, J. W. and Were, K. E. (1990). Effect of dietary tryptophan on plasma and brain tryptophan, brain serotonin, and brain 5 hydroxyindoleacetic acid in rainbow trout. Journal of Nutritional Biochemistry **1**(1): 49-54.

Johnston, W. L. and Glanville, N. T. (1992). Effect of feeding and fasting on plasma tryptophan and tryptophan to large neutral amino-acid ratio, and on brain-serotonin turnover in rainbow-trout, *Oncorhynchus mykiss*. Fish Physiology and Biochemistry **10**(1): 11-22.

Jones, K. A. (1989). The palatability of amino acids and related compounds to rainbow trout, *Oncorhynchus mykiss*. Journal of Fish Biology **34**(1): 149-160.

Jørgensen, E. H. and Jobling, M. Feeding and growth of exercised and unexercised juvenile Atlantic salmon in freshwater, and performance after transfer to seawater. Aquaculture International **2**(3): 154-164.

Kageyama, K., Tozawa, F., Horiba, N., Watanobe, H. and Suda, T. (1998). Serotonin stimulates corticotropin-releasing factor gene expression in the hypothalamic paraventricular nucleus of conscious rats. Neuroscience Letters **243**(1–3): 17-20.

Kalyanasundaram, S. and Ramanamurthy, P. S. (1983). Utilization of tyrosine and tryptophan for protein synthesis by undernourished developing rat brain. Neurochemical Research **8**(11): 1471-1480.

Katersky, R. S. and Carter, C. G. (2005). Growth efficiency of juvenile barramundi, *Lates calcarifer*, at high temperatures. Aquaculture **250**(3-4): 775-780.

Kaushik, S. J., Cravedi, J. P., Lalles, J. P., Sumpter, J., Fauconneau, B. and Laroche, M. (1995). Partial or total replacement of fish-meal by soybean protein on growth, protein-

utilization, potential estrogenic or antigenic effects, cholesterolemia and flesh quality in rainbow-trout, *Oncorhynchus mykiss*. Aquaculture **133**(3-4): 257-274.

Keast, A. and Eadie, J. M. (1985). Growth depensation in year-0 largemouth bass: the influence of diet. Transactions of the American Fisheries Society **114**(2): 204-213.

Kiser, D., SteemerS, B., Branchi, I. and Homberg, J. R. (2012). The reciprocal interaction between serotonin and social behaviour. Neuroscience & Biobehavioral Reviews **36**(2): 786-798.

Knowles, T. G., Brown, S. N., Warriss, P. D., Lines, J., Tinarwo, A. and Sendon, M. (2008). Effect of electrical stunning at slaughter on the quality of farmed turbot (*Psetta maxima*). Aquaculture Research **39**(16): 1731-1738.

Koch, H. J. A. (1982). Salmonid Smoltification Hemoglobin changes with size in the Atlantic salmon (*Salmo salar* L.). Aquaculture **28**(1): 231-240.

Koebele (1985). Growth and size hierarchy effect: an experimental assessment of three proposed mechanisms; activity differences, disproportional food acquisition, physiological stress. Environmental Biology of Fishes **12**: 181-188.

Koolhaas, J. M., de Boer, S. F., Buwalda, B. and van Reenen, K. (2007). Individual variation in coping with stress: a multidimensional approach of ultimate and proximate mechanisms. Brain, Behavior and Evolution **70**(4): 218-226.

Koolhaas, J. M., Korte, S. M., De Boer, S. F., Van Der Vegt, B. J., Van Reenen, C. G., Hopster, H., De Jong, I. C., Ruis, M. A. W. and Blokhuis, H. J. (1999). Coping styles in animals: current status in behavior and stress-physiology. Neuroscience and Biobehavioral Reviews **23**(7): 925-935.

Kravitz, E. A. (2000). Serotonin and aggression: insights gained from a lobster model system and speculations on the role of amine neurons in a complex behavior. Journal of Comparative Physiology A **186**(3): 221-238.

Lahti, K., Laurila, A., Enberg, K. and Piironen, J. (2001). Variation in aggressive behaviour and growth rate between populations and migratory forms in the brown trout, *Salmo trutta*. Animal Behaviour **62**: 935-944.

Larson, E. T., O'Malley, D. M. and Melloni, R. H. (2006). Aggression and vasotocin are associated with dominant-subordinate relationships in zebrafish. Behavioural Brain Research **167**(1): 94-102.

Leibowitz, S. F. and Alexander, J. T. (1998). Hypothalamic serotonin in control of eating behavior, meal size, and body weight. Biological Psychiatry **44**(9): 851-864.

Lema, S. C. and Nevitt, G. A. (2004). Exogenous vasotocin alters aggression during agonistic exchanges in male Amargosa River pupfish (*Cyprinodon nevadensis amargosae*). Hormones and Behavior **46**(5): 628-637.

Leopoldo, J., Laranja, Q., Jr., Quinitio, E. T., Catacutan, M. R. and Coloso, R. M. (2010). Effects of dietary L-tryptophan on the agonistic behavior, growth and survival of juvenile mud crab *Scylla serrata*. Aquaculture **310**(1-2): 84-90.

Lepage, O., Larson, E. T., Mayer, I. and Winberg, S. (2005). Serotonin, but not melatonin, plays a role in shaping dorninant-subordinate relationships and aggression in rainbow trout. Hormones and Behavior **48**(2): 233-242.

Lepage, O., Tottmar, O. and Winberg, S. (2002). Elevated dietary intake of L-tryptophan counteracts the stress-induced elevation of plasma cortisol in rainbow trout (*Oncorhynchus mykiss*). Journal of Experimental Biology **205**(23): 3679-3687.

Lepage, O., Vilchez, I. M., Pottinger, T. G. and Winberg, S. (2003). Time-course of the effect of dietary L-tryptophan on plasma cortisol levels in rainbow trout, *Oncorhynchus mykiss*. Journal of Experimental Biology **206**(20): 3589-3599.

Li, P., Mai, K., Trushenski, J. and Wu, G. (2009). New developments in fish amino acid nutrition: towards functional and environmentally oriented aquafeeds. Amino Acids **37**(1): 43-53.

Lijalad, M. and Powell, M. D. (2009). Effects of lower jaw deformity on swimming performance and recovery from exhaustive exercise in triploid and diploid Atlantic salmon Salmo salar L. Aquaculture **290**(1): 145-154.

Lin, X., Volkoff, H., Narnaware, Y., Bernier, N., J, Peyon, P. and Peter, R. E., P (2000). Brain regulation of feeding behavior and food intake in fish. Comparative Biochemistry and Physiology Part A(126): 415-434.

Lookingland K, J., Shannon N, J., Chapin D, S. and Moore K, E. (1986). Exogenous tryptophan increases synthesis, storage, and intraneuronal metabolism of 5-hydroxytryptamine in the rat hypothalamus. Journal of Neurochemistry **47**(1): 205-212.

Lovell, T. (1998). Nutrition and Feeding of Fish. New York, Springer US.

MacKinnon, M. (1985). Barramundi breeding and culture in Thailand. Queensland Dept. Primary Industries Study Tour Report, 1-21 June 1982. Sohgkhla, Thailand.

Madison, B. N., Tavakoli, S., Kramer, S. and Bernier, N. J. (2015). Chronic cortisol and the regulation of food intake and the endocrine growth axis in rainbow trout. J Endocrinol **226**(2): 103-119.

Madsen, S. S. (1990). The role of cortisol and growth hormone in seawater adaptation and development of hypoosmoregulatory mechanisms in sea trout parr (*Salmo trutta trutta*). General and Comparative Endocrinology **79**(1): 1-11.

McCormick, S. D. (1996). Effects of growth hormone and insulin-like growth factor I on salinity tolerance and gill Na+, K+-ATPase in atlantic salmon (*salmo salar*): interaction with cortisol. General and Comparative Endocrinology **101**(1): 3-11.

McCormick, S. D., Farrell, A. and Brauner, C. (2013). Fish Physiology: Euryhaline Fishes, Acedemic Press.

McCormick, S. D., Shrimpton, J. M., Carey, J. B., O'Dea, M. F., Sloan, K. E., Moriyama, S. and Björnsson, B. T. (1998). Repeated acute stress reduces growth rate of Atlantic salmon parr and alters plasma levels of growth hormone, insulin-like growth factor I and cortisol. Aquaculture **168**(1–4): 221-235.

McCormick, S. D., Shrimpton, J. M., Moriyama, S. and Björnsson, B. T. (2007). Differential hormonal responses of Atlantic salmon parr and smolt to increased daylength: A possible developmental basis for smolting. Aquaculture **273**(2–3): 337-344.

Medeiros, L. R., Cartolano, M. C. and McDonald, M. D. (2014). Crowding stress inhibits serotonin 1A receptor-mediated increases in corticotropin-releasing factor mRNA expression and adrenocorticotropin hormone secretion in the Gulf toadfish. Journal of Comparative Physiology B **184**(2): 259-271.

Mehlman, P. T., Higley, J. D., Faucher, I., Lilly, A. A., Taub, D. M., Vickers, J., Suomi, S. J. and Linnoila, M. (1994). Low CSF 5-HIAA concentrations and severe aggression and impaired impulse control in nonhuman-primates. American Journal of Psychiatry **151**(10): 1485-1491.

Metcalfe, N. B. (1989). Differential response to a competitor by Atlantic salmon adopting alternative life-history strategies. Proc R Soc Lond B Biol Sci **236**(1282): 21-27.

Metcalfe, N. B., Huntingford, F. A., Graham, W. D. and Thorpe, J. E. (1989). Early social status and the development of life-history strategies in Atlantic salmon. Proc R Soc Lond B Biol Sci **236**(1282): 7-19.

Miczek, K. A., Hussain, S. and Faccidomo, S. (1998). Alcohol-heightened aggression in mice: attenuation by 5-HT1A receptor agonists. Psychopharmacology **139**(1): 160-168.

Mommsen, T. P., Vijayan, M. M. and Moon, T. W. (1999). Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Reviews in Fish Biology and Fisheries **9**(3): 211-268.

Mork, O., I., Bjerkeng, B. and Rye, M. (1999). Aggressive interactions in pure and mixed groups of juvenile farmed and hatchery-reared wild Atlantic salmon, *Salmo salar* L. in relation to tank substrate. Aquaculture Research **30**(8): 571-578.

Moyer, K. E. (1971). The Physiology of Hostility. Chicago.

Nakagami, Y., Suda, T., Yajima, F., Ushiyama, T., Tomori, N., Sumitomo, T., Demura, H. and Shizume, K. (1986). Effects of serotonin, cyproheptadine and reserpine on corticotropin-releasing factor release from the rat hypothalamus in vitro. Brain Research **386**(1–2): 232-236.

Naylor, R. L., Goldburg, R. J., Primavera, J. H., Kautsky, N., Beveridge, M. C. M., Clay, J., Folke, C., Lubchenco, J., Mooney, H. and Troell, M. (2000). Effect of aquaculture on world fish supplies. Nature **405**(6790): 1017-1024.

Naylor, R. L., Hardy, R. W., Bureau, D. P., Chiu, A., Elliott, M., Farrell, A. P., Forster, I., Gatlin, D. M., Goldburg, R. J., Hua, K. and Nichols, P. D. (2009). Feeding aquaculture in an era of finite resources. Proceedings of the National Academy of Sciences **106**(36): 15103-15110.

Nelson, R. J. and Chiavegatto, S. (2001). Molecular basis of aggression. Trends in Neurosciences **24**(12): 713-719.

Newman, S. W. (1999). The medial extended amygdala in male reproductive behavior - A node in the mammalian social behavior network. Advancing from the Ventral Striatum to the Extended Amygdala: Implications for Neuropsychiatry and Drug Abuse: In Honor of Lennart Heimer. J. F. McGinty. **877:** 242-257.

Noble, C., Kadri, S., Mitchell, D. F. and Huntingford, F. A. (2008). Growth, production and fin damage in cage-held 0+ Atlantic salmon pre-smolts (*Salmo salar* L.) fed either a) on-demand, or b) to a fixed satiation-restriction regime: Data from a commercial farm. Aquaculture **275**(1-4): 163-168.

NRC (1992). Nutrient Requirements of Fish, National Academies Press.

O'Flynn, F. M., McGeachy, S. A., Friars, G. W., Benfey, T. J. and Bailey, J. K. (1997). Comparisons of cultured triploid and diploid Atlantic salmon(Salmo salar L.). ICES Journal of Marine Science **54**(6): 1160-1165.

Oliveira, R. F., Silva, A. and Canario, A. V. M. (2009). Why do winners keep winning? Androgen mediation of winner but not loser effects in cichlid fish. Proceedings of the Royal Society of London. Series B, Biological Sciences **276**(1665): 2249-2256.

Olla B, L. and Samet, C. (1974). Fish-to-fish attraction and the facilitation of feeding behaviour as mediated by visual stimuli in striped mullet. Journal of the Fisheries Research Board of Canada **31**: 1621 - 1630.

Olsen, K. H., Petersson, E., Ragnarsson, B., Lundqvist, H. and Jarvi, T. (2004). Downstream migration in Atlantic salmon (*Salmo salar*) smolt sibling groups. Canadian Journal of Fisheries and Aquatic Sciences **61**(3): 328-331.

Ortega, V. A., Lovejoy, D. A. and Bernier, N. J. (2013). Appetite-suppressing effects and interactions of centrally administered corticotropin-releasing factor, urotensin I and serotonin in rainbow trout (*Oncorhynchus mykiss*). Frontiers in Neuroscience **7**.

Ortega, V. A., Renner, K. J. and Bernier, N. J. (2005). Appetite-suppressing effects of ammonia exposure in rainbow trout associated with regional and temporal activation of brain monoaminergic and CRF systems. J Exp Biol **208**(Pt 10): 1855-1866.

Overli, O., Harris, C. and Winberg, S. (1999). Short-term effects of fights for social dominance and the establishment of dominant-subordinate relationships on brain monoamines and cortisol in rainbow trout. Brain Behavior and Evolution **54**(5): 263 - 275.

Overli, O., Winberg, S., Damsgard, B. and Jobling, M. (1998). Food intake and spontaneous swimming activity in Arctic char (*Salvelinus alpinus*): role of brain serotonergic activity and social interactions. Canadian Journal of Zoology-Revue Canadienne De Zoologie **76**(7): 1366-1370.

Pankhurst, N. W., Ludke, S. L., King, H. R. and Peter, R. E. (2008). The relationship between acute stress, food intake, endocrine status and life history stage in juvenile farmed Atlantic salmon, *Salmo salar*. Aquaculture **275**(1–4): 311-318.

Papoutsoglou, S. E., Karakatsouli, N. and Chiras, G. (2005). Dietary l-tryptophan and tank colour effects on growth performance of rainbow trout (*Oncorhynchus mykiss*) juveniles reared in a recirculating water system. Aquacultural Engineering **32**(2): 277-284.

Parazo, M. M., Avila, E. M. and Reyes, D. M. J. (1991). Size and weight dependent cannibalism in hatchery bred sea bass (*Lates calcarifer*). Journal of Applied Ichthyology **7**: 1-7.

Pardridge, W. M. and Oldendorf, W. H. (1977). Transport of metabolic substrates through blood-brain-barrier. Journal of Neurochemistry **28**(1): 5-12.

Parent, A. (1984). Functional Anatomy and Evolution of Monoaminergic Systems. American Zoologist **24**(3): 783-790.

Park, J.-Y., Myung, S.-W., Kim, I.-S., Choi, D.-K., Kwon, S.-J. and Yoon, S.-H. (2013). Simultaneous Measurement of Serotonin, Dopamine and Their Metabolites in Mouse Brain Extracts by High-Performance Liquid Chromatography with Mass Spectrometry Following Derivatization with Ethyl Chloroformate. Biological and Pharmaceutical Bulletin **36**(2): 252-258.

Pelis, R. M. and McCormick, S. D. (2003). Fin development in stream- and hatchery-reared Atlantic salmon. Aquaculture **220**(1-4): 525-536.

Pérez-Maceira, J. J., Otero-Rodiño, C., Mancebo, M. J., Soengas, J. L. and Aldegunde, M. (2016). Food intake inhibition in rainbow trout induced by activation of serotonin 5-HT2C

receptors is associated with increases in POMC, CART and CRF mRNA abundance in hypothalamus. Journal of Comparative Physiology B **186**(3): 313-321.

Pérez Maceira, J. J., Mancebo, M. J. and Aldegunde, M. (2014). The involvement of 5-HT-like receptors in the regulation of food intake in rainbow trout (*Oncorhynchus mykiss*). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **161**: 1-6.

Perreault, H. A. N., Semsar, K. and Godwin, J. (2003). Fluoxetine treatment decreases territorial aggression in a coral reef fish. Physiology & Behavior **79**(4–5): 719-724.

Pervin, L. and John, O. p. (1991). Handbook of Personality:Theory and Research, Guidford Press.

Pfeiffer, T. J. and Freeman, D. W. (2004). Effects of water temperature on sock grading of sub-marketable channel catfish, *ictalurus punctatus*, in earthen ponds. Journal of Applied Aquaculture **16**(1-2): 95-103.

Pickering, A. D. (1993). Growth and stress in fish production. Aquaculture **111**(1–4): 51-63.

Pickering, A. D. (1998). Stress responses of farmed fish. Biology of farmed fish. K. Black and A. D. Pickering. Sheffield, Sheffield Academic Press**:** 222-238.

Pinto, W., Aragao, C., Soares, F., Dinis, M. T. and Conceicao, L. E. C. (2007). Growth, stress response and free amino acid levels in Senegalese sole (*Solea senegalensis* Kaup 1858) chronically exposed to exogenous ammonia. Aquaculture Research **38**(11): 1198-1204.

Pottinger, T. G. and Carrick, T. R. (2001). Stress responsiveness affects dominant-subordinate relationships in rainbow trout. Hormones and Behavior **40**(3): 419-427.

Pottinger, T. G., Moran, T. A. and Morgan, J. A. W. (1994). Primary and secondary indexes of stress in the progeny of rainbow trout (*Oncorhynchus mykiss*) selected for high and low responsiveness to stress. Journal of Fish Biology **44**(1): 149-163.

Qin, J. and Fast, A. W. (1996). Size and feed dependent cannibalism with juvenile snakehead, *Channa striatus*. Aquaculture **144**(4): 313-320.

Qin, J. G., Mittiga, L. and Ottolenghi, F. (2004). Cannibalism reduction in juvenile barramundi, *Lates calcarifer*, by providing refuges and low light. Journal of the World Aquaculture Society **35**(1): 113-118.

Rey, S., Digka, N. and MacKenzie, S. (2015). Animal Personality Relates to Thermal Preference in Wild-Type Zebrafish, Danio rerio. Zebrafish **12**(3): 243-249.

Ribeiro, F., F and Qin, J., G (2013). Modelling Size-Dependent Cannibalism in Barramundi Lates calcarifer: Cannibalistic Polyphenism and Its Implication to Aquaculture. PLOS ONE **8**(12): e82488.

Ribeiro, F. F., Forsythe, S. and Qin, J. G. (2015). Dynamics of intracohort cannibalism and size heterogeneity in juvenile barramundi (*Lates calcarifer*) at different stocking densities and feeding frequencies. Aquaculture **444**: 55-61.

Ribeiro, F. F. and Qin, J. G. (2016). Bioenergetics of cannibalism in juvenile barramundi *Lates calcarifer* (Bloch): exploring growth advantage of fish fed live prey and formulated diet. Aquaculture Research **47**(7): 2324-2333.

Riddell, B. E. and Swain, D. P. (1991). Competition between hatchery and wild coho salmon (*Oncorhynchus kisutch*) - Genetic variation for agonistic behaviour in newly emerged wild fry. Aquaculture **98**(1-3): 161-172.

Rollin, X., Mambrini, M., Abboudi, T., Larondelle, Y. and Kaushik, S. J. (2003). The optimum dietary indispensable amino acid pattern for growing Atlantic salmon (*Salmo salar* L.) fry. British Journal of Nutrition **90**(5): 865-876.

Ronan, P. J. and Summers, C. H. (2011). Molecular signaling and translational significance of the corticotropin releasing factor system. Progress in molecular biology and translational science **98**: 235-292.

Ruibal, C., Soengas, J. L. and Aldegunde, M. (2002). Brain serotonin and the control of food intake in rainbow trout (*Oncorhynchus mykiss*): effects of changes in plasma glucose levels. Journal of Comparative Physiology (A) Neuroethology Sensory Neural and Behavioral Physiology **188**(6): 479-484.

Rutte, C., Taborsky, M. and Brinkhof, M. W. (2006). What sets the odds of winning and losing? Trends in Ecolgy & Evolution **21**(1): 16-21.

Saunders, R. L. (1965). Adjustment of buoyancy in young Atlantic salmon and brook trout by changes in swimbladder volume. Journal of the Fisheries Research Board of Canada **22**(2): 335-352.

Savage, J. and Hobsbawn, P. (2015). Australian fisheries and aquaculture statistics 2014. Fisheries Research and Development Corporation.

Schaechter, J. D. and Wurtman, R. J. (1989). Tryptophan availability modulates serotonin release from rat hypothalamic slices. Journal of Neurochemistry **53**(6): 1925-1933.

Schreck C. B., Olla B. L. and Davis, M. W. (1997). Behavioral responses to stress. Fish Stress and Health in Aquaculture. Iwama G. K., Pickering A. D., Sumpter J. P. and S. C. B. Cambridge, Cambridge University Press**:** 145-170.

Schreck CB, Olla BL and MW., D. (1997). Fish stress and health in aquaculture. Iwama GK, Pickering AD, Sumpter JP and Schreck CB, Cambridge University Press. **:** 145–170.

Schulkin, J., Gold, P. W. and McEwen, B. S. (1998). Induction of corticotropin-releasing hormone gene expression by glucocorticoids: implication for understanding the states of fear and anxiety and allostatic load. Psychoneuroendocrinology **23**(3): 219-243.

Sih, A., Bell, A. and Johnson, J. C. (2004). Behavioral syndromes: an ecological and evolutionary overview. Trends Ecol Evol **19**(7): 372-378.

Sink, T. D., Kumaran, S. and Lochmann, R. T. (2007). Development of a whole-body cortisol extraction procedure for determination of stress in golden shiners, *Notemigonus crysoleucas*. Fish Physiology and Biochemistry **33**(3): 189-193.

Sloman, K. A., Motherwell, G., O'Connor, K. I. and Taylor, A. C. (2000). The effect of social stress on the Standard Metabolic Rate (SMR) of brown trout, *Salmo trutta*. Fish Physiology and Biochemistry **23**(1): 49-53.

Smith, C. and Reay, P. (1991). Cannibalism in teleost fish. Reviews in Fish Biology and Fisheries **1**(1): 41-64.

Smith, T., M and Smith, R., L (2009). Elements of Ecology. San Francisco, Pearson Benjamin Cummings.

Soengas, J. L. (2014). Contribution of glucose- and fatty acid sensing systems to the regulation of food intake in fish. A review. General and Comparative Endocrinology **205**: 36-48.

Specker, J. L. and Schreck, C. B. (1982). Changes in plasma corticosteroids during smoltification of coho salmon, *Oncorhynchus kisutch*. General and Comparative Endocrinology **46**(1): 53-58.

Spence, J. R. and Carcamo, H., A (1991). Effects of cannibalism and intraguild predation on pondskaters (Gerridae). Oikos **62**(3): 333-341.

Sperry, T. S., Thompson, C. K. and Wingfield, J. C. (2003). Effects of acute treatment with 8-OH-DPAT and fluoxetine on aggressive behaviour in male song sparrows (*Melospiza melodia morphna*). Journal of Neuroendocrinology **15**(2): 150-160.

Stead, S. M., Houlihan, D. F., McLay, H. A. and Johnstone, R. (1996). Effect of ration and seawater transfer on food consumption and growth of Atlantic salmon (*Salmo salar*) smolts. Canadian Journal of Fisheries and Aquatic Sciences **53**(5): 1030-1037.

Stradmeyer, L. (1994). Survival, growth and feeding of Atlantic salmon, *Salmo salar* L., smolts after transfer to sea water in relation to the failed smolt syndrome. Aquaculture Research **25**(1): 103-112.

Summers, C. H., Korzan, W. J., Lukkes, J. L., Watt, M. J., Forster, G. L., Overli, O., Hoglund, E., Larson, E. T., Ronan, P. J., Matter, J. M., Summers, T. R., Renner, K. J. and Greenberg, N. (2005). Does serotonin influence aggression? Comparing regional activity before and during social interaction. Physiological and Biochemical Zoology **78**(5): 679-694.

Summers, C. H. and Winberg, S. (2006). Interactions between the neural regulation of stress and aggression. Journal of Experimental Biology **209**(23): 4581-4589.

Suomi, S. J. (2006). Risk, resilience, and gene x environment interactions in rhesus monkeys. Ann N Y Acad Sci: 52-62.

Symons, P. E. K. (1968). Increase in aggression and in strength of the social hierarchy among juvenile Atlantic salmon deprived of food. Journal of the Fisheries Research Board of Canada **25**(11): 2387-2401.

Taylor, E. B. and Larkin, P. A. (1986). Current response and agonistic behaviour in newly emerged fry of chinook salmon, *Oncorhynchus tshawytscha*, from ocean-type and stream-type populations. Canadian Journal of Fisheries and Aquatic Sciences **43**(3): 565-573.

Taylor, M. (1976). Effects of L-tryptophan and L-methionine on activity in the rat. British Journal of Pharmacology **58**(1): 117-119.

Thodesen, J., Grisdale-Helland, B., Helland, S. J. and Gjerde, B. (1999). Feed intake, growth and feed utilization of offspring from wild and selected Atlantic salmon (*Salmo salar*). Aquaculture **180**(3–4): 237-246.

Thornqvist, P. O., Hoglund, E. and Winberg, S. (2015). Natural selection constrains personality and brain gene expression differences in Atlantic salmon (*Salmo salar*). J Exp Biol **218**(Pt 7): 1077-1083.

Tinbergen, N. (1951). The Study of Instinct. Oxford, Oxford University Press.

Torres, I. L. S., Gamaro, G. D., Vasconcellos, A. P., Silveira, R. and Dalmaz, C. (2002). Effects of chronic restraint stress on feeding behavior and on monoamine levels in different brain structures in rats. Neurochemical Research **27**(6): 519-525.

Trenzado, C. E., Carrick, T. R. and Pottinger, T. G. (2003). Divergence of endocrine and metabolic responses to stress in two rainbow trout lines selected for differing cortisol responsiveness to stress. General and Comparative Endocrinology **133**(3): 332-340.

Tucker, J. W., Jr., John Russell, D. and Rimmer, M. A. (2002). Barramundi culture: A success story for aquaculture in Asia and Australia. World Aquaculture **33**(3): 53-59,72.

Usher, M. L., Talbot, C. and Eddy, F. B. (1988). Drinking in Atlantic salmon smolts transferred to seawater and the relationship between drinking and feeding. Aquaculture **73**(1): 237-246.

Usher, M. L., Talbot, C. and Eddy, F. B. (1991). Effects of transfer to seawater on growth and feeding in Atlantic salmon smolts (*Salmo salar* L.). Aquaculture **94**(4): 309-326.

Valassi, E., Scacchi, M. and Cavagnini, F. (2008). Neuroendocrine control of food intake. Nutr Metab Cardiovasc Dis **18**(2): 158-168.

Vale, W., Spiess, J., Rivier, C. and Rivier, J. (1981). Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. Science **213**(4514): 1394-1397.

van Erp, A. M. M. and Miczek, K. A. (1997). Increased aggression after ethanol self-administration in male resident rats. Psychopharmacology **131**(3): 287-295.

Vijayan, M. M. and Leatherland, J. F. (1990). High stocking density affects cortisol secretion and tissue distribution in brook charr, *Salvelinus fontinalis*. Journal of Endocrinology **124**(2): 311-318.

Vijayan, M. M., Pereira, C., Grau, E. G. and Iwama, G. K. (1997). Metabolic responses associated with confinement stress in tilapia: The role of cortisol. Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology **116**(1): 89-95.

Wang, T. C. and Fuller, M. F. (1989). The optimum dietary amino acid pattern for growing pigs. British Journal of Nutrition **62**: 77-89.

Wendelaar Bonga, S. E. (1997). The stress response in fish. Physiological Reviews **77**(3): 591-625.

Wilkinson, R. J., Paton, N. and Porter, M. J. R. (2008). The effects of pre-harvest stress and harvest method on the stress response, rigor onset, muscle pH and drip loss in barramundi (*Lates calcarifer*). Aquaculture **282**(1/4): 26-32.

Wilson, D. S., Coleman, K., Clark, A. B. and Biederman, L. (1993). Shy-bold continuum in pumpkinseed sunfish (*Lepomis gibbosus*): An ecological study of a psychological trait. Journal of Comparative Psychology **107**(3): 250-260.

Wilson, E. O. (1975). Sociobiology. Cambridge, USA, Harvard University Press.

Wilson, R. P. (2002). Protein and amino acids. Fish Nutrition. J. E. Halver and R. W. Hardy. San Diego, USA, Elsevier Science**:** 144-179.

Winberg, S., Carter, C. G., Mccarthy, I. D., He, Z. Y., Nilsson, G. E. and Houlihan, D. F. (1993). Feeding rank and brain serotonergic activity in rainbow trout, *Oncorhynchus mykiss*. Journal of Experimental Biology **179**(1): 197-211.

Winberg, S., Myrberg, A. A. and Nilsson, G. E. (1996). Agonistic interactions affect brain serotonergic activity in an acanthopterygiian fish: The bicolor damselfish (*Pomacentrus partitus*). Brain Behavior and Evolution **48**(4): 213-220.

Winberg, S., Nilsson, A., Hylland P., Soderstom V. and Nilsson, G. E. (1997). Serotonin as a regulator of hypothalamic-pituitary-interrenal activity in teleost fish. Neuroscience Letters **230**(2): 113-116.

Winberg, S., Nilsson G, E., . and Olsen K, H. (1992). The Effect of Stress and Starvation on Bratn Serotonin Utilization in Arctic Charr (*Salvelinus Alpinus*). Journal of Experimental Biology **165**(1): 229-239.

Winberg, S. and Nilsson, G. E. (1993a). Roles of brain monoamine neurotransmitters in agonistic behaviour and stress reactions, with particular reference to fish. Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology **106**(3): 597-614.

Winberg, S. and Nilsson, G. E. (1993b). Time course of changes in brain serotonergic activity and brain tryptophan levels in dominant and subordinate juvenile arctic charr. Journal of Experimental Biology **179**(1): 181-195.

Winberg, S., Overli, O. and Lepage, O. (2001). Suppression of aggression in rainbow trout (*Oncorhynchus mykiss*) by dietary L-tryptophan. Journal of Experimental Biology **204**(22): 3867-3876.

Winberg, S. and Thörnqvist, P.-O. (2016). Role of brain serotonin in modulating fish behavior. Current Zoology **62**(3): 317.

Wolkers, C. P., Serra, M., Hoshiba, M. A. and Urbinati, E. C. (2012). Dietary L-tryptophan alters aggression in juvenile matrinxa *Brycon amazonicus*. Fish Physiol Biochem **38**(3): 819-827.

Wootton, R. J. (1990). Ecology of Teleost Fishes. New York, Chapman & Hall.

Wu, G. (2009). Amino acids: metabolism, functions, and nutrition. Amino Acids **37**(1): 1-17.

Wurtman, R. J. (1988). Presynaptic control of release of amine neurotransmitters by precursor levels. NIPS (Am. Physiol. Soc.) **3**: 158 - 163.

Wurtman, R. J. (1994). Effects of Nutrients on Neurotransmitter Release. Food components to enhance performance: An evaluation of potential performance-enhancing food components for operational rations. B. M. Marriott. Washington (DC), National Academies Press (US).

Yao, K., Fang, J., Yin, Y., Feng, Z.-M., Tang, Z.-R. and Wu, G. (2011). Tryptophan metabolism in animals: important roles in nutrition and health. Front Biosci **3**: 286-297.

Yeh, S.-R., Musolf, B. E. and Edwards, D. H. (1997). Neuronal adaptations to changes in the social dominance status of crayfish. The Journal of Neuroscience **17**(2): 697-708.

Yeh, S. R., Fricke, R. A. and Edwards, D. H. (1996). The effect of social experience on serotonergic modulation of the escape circuit of crayfish. Science **271**(5247): 366-369.

Young, G., Björnsson, B. T., Prunet, P., Lin, R. J. and Bern, H. A. (1989). Smoltification and seawater adaptation in coho salmon (*Oncorhynchus kisutch*): Plasma prolactin, growth hormone, thyroid hormones, and cortisol. General and Comparative Endocrinology **74**(3): 335-345.